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March 16, 2006

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March 16, 2006 Timothy S. Corder
Date

MAIL STOP APPEAL BRIEF-PATENTS
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Alexandria, VA 22313-1450

Re: U. S. Patent Application Serial No. 10/790,658 entitled "*R(-)-Desmethylselegiline* and Its Use to Treat Immune System Dysfunction" by Cheryl D. Blume, *et al.* (Our Ref: SOM700/4-4CIP2CON2DIVUS/13004)

Dear Sir:

Enclosed for filing in the above-referenced patent application are the following:

1. Appeal Brief in triplicate (33 pages each); and

2. Postcard.

If additional fees are due related to this filing, the Commissioner is authorized to appropriately deduct the requisite amount from Vinson & Elkins L.L.P. Deposit Account No. 22-0365/SOM700/4-4CIP2CON2DIVUS/13004.

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of:
Cheryl D. Blume *et al.*

Serial No.: 10/790,658

Filed: March 1, 2004

For: R(-)-DESMETHYLSELEGILINE AND ITS USE TO TREAT IMMUNE SYSTEM DYSFUNCTION

Group Art Unit: 1615

Examiner: L.S. Channavajjala

Atty. Dkt. No.: SOM700/4-4CIP2CON2DIVUS/13004

Confirmation No.: 9575

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March 16, 2006 /Timothy S. Corder/
Date Timothy S. Corder

APPEAL BRIEF

MAIL STOP APPEAL BRIEF-PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In support of the appeal from the second rejection dated May 17, 2005, Appellant now submits this Brief.

I. REAL PARTY IN INTEREST

The real party in interest of the patent application that is the subject of this appeal is the assignee, Somerset Pharmaceuticals, Inc.

II. RELATED APPEALS AND INTERFERENCES

Appeals are pending in the following two applications, which are also assigned to Somerset Pharmaceuticals, Inc..

1. Serial No. 10/885,221, Anthony R. DiSanto, filed July 6, 2004.

2. Serial No. 10/806,494, Mark G. Resnick, filed March 3, 2004.

This application does not does not claim priority to either of the above applications.

III. STATUS OF CLAIMS

Claims 26 and 34-62 are pending in the instant application and are the subject of this Appeal. Claims 26 and 34-62 stand rejected by the Examiner.

IV. STATUS OF AMENDMENTS

None

V. SUMMARY OF CLAIMED SUBJECT MATTER

Appellant's application contains independent claims 26, 43 and 46. Claim 26 recites a method of treating a condition in a mammal produced by immune system dysfunction that is associated with reduced levels of gamma-interferon ("γ-interferon") production by administering to the mammal the R(-)-enantiomer of desmethylselegiline, or a pharmaceutically acceptable salt thereof (Specification, p.8, ll.21-22), at a daily dose of at least 0.015 mg/kg of the mammal's body weight, calculated on the basis of the free secondary amine (*Id.* at p.9, ll.12-16), wherein such administration leads to an increase in γ-interferon production in the mammal. *See, e.g., id.* at p.39, ll. 12-14. The daily dose may be administered in either a single or multiple dosage regimen. *Id.* at p.9, l.22.

Claim 43 recites a method of treating a condition in a mammal produced by immune system dysfunction caused by cancer chemotherapy which is associated with reduced levels of γ-interferon production by administering to the mammal the R(-) enantiomer of desmethylselegiline, or pharmaceutically acceptable salts thereof (*Id.*, p.8, ll.21-22), at a daily dose of at least 0.015 mg/kg of the mammal's body weight, calculated on the basis of the free secondary amine (*Id.* at p.9, ll.12-16), wherein such administration leads to an increase in γ-interferon production in the mammal. *See, e.g., id.* at p.39, ll. 12-14. The daily dose may be administered in either a single or multiple dosage regimen. *Id.* at p.9, l.22.

Claim 46 recites a method of treating a condition in a mammal produced by immune system dysfunction that is associated with reduced levels of γ-interferon production by administering to the mammal the R(-) enantiomer of desmethylselegiline, or a pharmaceutically acceptable salt thereof (*Id.*, p.8, ll.21-22), wherein such

administration leads to an increase in γ-interferon production in the mammal. *See, e.g.*, *id.* at p.39, ll. 12-14.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. Whether claims 26 and 34-62 are unpatentable under 35 U.S.C. § 112 for lack of enablement.

2. Whether claims 26 and 34-62 are unpatentable under 35 U.S.C. § 103(a) as obvious over Borbe (J. Neural. Transm. Suppl. 1990) in view of Barton et al (J. Neurooncol.) and Balsa et al (Biochem. Pharmacol. 1987).

VII. ARGUMENT

A. The Rejection Based on 35 U.S.C. § 112, First Paragraph, for Lack of Enablement Should Be Overturned.

1. Claims 26 and 34-62

Claims 26 and 34-62 stand rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement. The Examiner argues that the "claims are broad as they encompass a number of 'conditions' that are stimulated or caused by immune dysfunction or immune deficiency." 5-17-05 Office Action, p. 2. The Examiner then proceeds to consider enablement of the claims in view of the *Wands* factors, and argues that the claims do not comply with the enablement requirement.

When evaluating enablement, the judicial standard specifies that a claimed invention is enabled if a person of skill in the art can make and use the invention without undue experimentation. MPEP § 2164.01 (discussing *In re Wands*, 858 F.2d 731 (Fed. Cir. 1988)). "In order to make a rejection, the Examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention." MPEP § 2164.04 (citing *In re Wright*, 999 F.2d 1557 (Fed. Cir. 1993)).

The Examiner has not met her burden of establishing a reasonable basis to question the enablement provided for the claimed subject matter. Appellant asserts that the pending claims are enabled because it is well within the skill of a person in the art to determine whether a condition produced by immune system dysfunction is associated with reduced levels of γ-interferon production, and whether administering the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof, leads to an increase in γ-interferon production. The underlying complexity of the immune system does not mean that the pending claims are not enabled, contrary to

the Examiner's unsupported conclusion. Measuring the levels of γ-interferon production in a mammal is well within the skill of one in the art, and does not require undue experimentation.

a. The Nature of the Invention Does Not Weigh Against Enablement

The Examiner argues that the "nature of invention is extremely complex in that it encompasses anticipating multiple complex diseases or disorders," and that the "breadth of the claims exacerbates the complex nature of the claims." 5-17-05 Office Action, p. 3. But whether or not the claims encompass multiple complex diseases or disorders is irrelevant to the question of enablement of the present claims, since one of skill in the art will clearly be able to understand and practice the scope of the pending claims without undue experimentation. Appellant believes that the Examiner is inappropriately focusing on the complex nature of immune system dysfunction to reject the claims, rather than on the actual scope of the claimed subject matter.

Whether a claim is enabled is determined by evaluating whether or not one of ordinary skill in the art would be able to make and use the claimed invention based on the disclosure. MPEP § 2164.01. Thus, the real questions with respect to enablement of the presently pending claims are: (1) whether one of skill in the art can identify conditions that fall within the genus of conditions produced by immune system dysfunction associated with reduced levels of γ-interferon production without undue experimentation; and (2) whether one of skill in the art can identify an increase in γ-interferon production in a mammal after administration of the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof without undue experimentation. The answer to both of these questions is yes.

Reduced levels of γ-interferon production, and the role such reduced levels play in immune system dysfunction, is clearly understood in the art. The reference cited in Appellant's Response to the Office Action dated October 21, 2004, filed January 21, 2005 (received by the USPTO on January 25, 2005), Billiau, A., *Interferon-γ: Biology and Role in Pathogenesis*, ADV. IMMUNOL. 62:61-130 (1996), clarifies the correlation of γ-interferon (IFN-γ) to conditions related to immune deficiency such as cancer and AIDS, as well as autoimmune diseases. The cytokine γ-interferon plays a central role in the immune system, and immune dysfunction related to γ-interferon has been recognized in both immune deficiency and autoimmune diseases:

> Medical interest in IFN-γ stems from awareness that a prominent target cell of IFN-γ, the macrophage, occupies a central position in the immune system. Adequate function of the IFN-γ/macrophage system is essential for natural as well as acquired resistance to infection and cancer. Malfunctioning of the system is recognized to be instrumental in inflammatory and autoimmune disease. *Id.* at 62.

Thus, although immune system function "is a complex interplay of several interleukins or chemokines," γ-interferon has a central position in the immune system and is involved in both immune deficiency and autoimmune diseases. 10-21-04 Office Action, p. 3.

The claimed subject matter is directed to the novel discovery that administration of R(-)-desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof leads to an increase in γ-interferon production. As stated in the specification, the ability of R(-)-desmethylselegiline to restore γ-interferon production supports the conclusion that this enantiomer of desmethylselegiline is able to treat certain conditions in a mammal produced by immune system dysfunction, *i.e.*, those that are associated with reduced levels of γ-interferon production. *See* Specification, Example 11, beginning on p.38.

Given that the malfunctioning of the IFN-γ/macrophage system is recognized to be instrumental in inflammatory and autoimmune diseases, the ability of R(-)-desmethylselegiline to restore IFN-γ production will "bolster a patient's normal immunological defenses [and] be beneficial in the treatment of a wide variety of acute and chronic diseases including cancer, AIDS, and both bacterial and viral infections." (*See Id.*, p.38, ll.12-14).

The Examiner ignores the well-defined scope of the claimed subject matter and focuses instead on the complexity of the potential underlying causes of the conditions produced by immune system dysfunction to reject the claims. But this argument is inappropriate for questioning the enablement of the pending claims, and ignores the fact that one of skill in the art will be able to routinely determine whether a mammal with a condition produced by immune system dysfunction has reduced levels of γ-interferon production, and whether administering the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof leads to an increase in γ-interferon production.

b. The State of the Art Does not Weigh Against Enablement

The Examiner argues that "a reduction in gamma-interferon does not necessarily result in immune system dysfunction," and therefore, "the described or claimed conditions may or may not be caused by gamma-interferon reduction leading to immune dysfuntion [sic]." 5-17-05 Office Action, pp. 3-4. Appellant asserts that this argument presented by the Examiner is irrelevant to the question of whether the pending claims are enabled.

The claims are specifically directed to methods of treating a condition in a mammal produced by immune system dysfunction that is associated with reduced levels of γ-interferon production. The scope of the claims is not directed to those conditions that have reduced γ-interferon production but no immune system dysfunction. In addition, the underlying mechanism causing the condition is not being claimed; instead the claims are simply directed to treating conditions produced by immune system dysfunction that are associated with reduced levels of γ-interferon production. Since the scope of the claims is clear to one of skill in the art, and would not require undue experimentation to practice, the pending claims are enabled.

c. The Specification Gives Sufficient Guidance to One of Skill in the Art to Practice the Claimed Subject Matter

The Examiner next argues that the claimed subject matter is not enabled because the "instant specification provides no guidance with respect to the procedure of administering instant composition to mammals for treating any or all of the disorders claimed." 5-17-05 Office Action, p. 4. But this is simply not true. The specification provides sufficient guidance to *one of skill in the art* for administering the instant composition to mammals for treating any or all of the disorders claimed. *See* Specification, p.9, l.8 to p.10, l.20. For example, on p.9, lines 8-21, the specification states:

> The optimal daily dose of R(-)DMS, S(+)DMS, or of a combination, such as a racemic mixture, of R(-)DMS and S(+)DMS, useful for the purposes of the present invention is determined by methods known in the art, *e.g.*, based on the severity of the disease or condition being treated, the condition of the subject to whom treatment is being given, the desired degree of therapeutic response, and the concomitant therapies being administered to the patient or animal. Ordinarily, however, the attending physician or veterinarian will administer an initial dose of at least about

> 0.015 mg/kg, calculated on the basis of the free secondary amine, with progressively higher doses being employed depending upon the route of administration and the subsequent response to the therapy... These guidelines further require that the actual dose be carefully titrated by the attending physician or veterinarian depending on the age, weight, clinical condition, and observed response of the individual patient or animal.

A person skilled in the art could readily determine the effective amount of R(-)-desmethylselegiline required to achieve a therapeutic effect based upon animal pharmacology and early phase clinical trials in humans, both of which are standard activities and practices in the pharmaceutical industry, and are permissible under MPEP § 2164.01(c). Enablement is determined from the perspective of one of skill in the art, and procedures for determining the effective amount of a particular drug to administer to a mammal are routine in this art.

The Examiner also argues that the specification "fails to provide any guidance or rationale showing that the claimed method is effective to completely treating [sic] any or all disorders produced by immune dysfunction, associated with reduced levels of gamma-IFN or to extrapolate the data provided to al [sic] immune dysfunction conditions, that are known to-date or yet to be discovered." 5-17-05 Office Action, p. 4.

Appellant submits that this is an improper rejection. The claims are drawn to 'treating a condition," and the Examiner has improperly inserted the limitation that the claimed method must "completely treat any and all disorders..." The methods of the claims are not described as total cures to any disease or condition, but rather as methods of treating. This subject matter is analogous to that in *In re Sichert*, 566 F.2d 1154, 1160, 196 USOQ 209, 212 (CCPA 1977), in which the appeal court used the analogy of over-the-counter ointment drugs which have the purpose of stimulating blood circulation. Use of an ointment to stimulate circulation and alleviate pain is not the same as treatment

directed at curing the disease (arthritis) that has caused the condition. Furthermore, Applicants are not required to demonstrate "full treatment" of a condition prior to filing an application. This issue has been addressed by the Federal Circuit:

> Testing for the full safety and effectiveness of a prosthetic device is more properly left to the Food and Drug Administration (FDA). Title 35 does not demand that such human testing occur within the confines of Patent and Trademark Office (PTO) proceedings. *Scott v. Finney* 32 USPQ2d 1115, 1120

Therefore, there is no requirement for patentability that the claimed methods be effective to treat any or all conditions produced by immune system dysfunction associated with reduced levels of γ-interferon production. As the MPEP acknowledges: "The presence of inoperative embodiments within the scope of a claim does not necessarily render a claim nonenabled. The standard is whether a skilled person could determine which embodiments that were conceived, but not yet made, would be inoperative or operative with expenditure of no more effort than is normally required in the art." MPEP § 2164.08(b). A person of skill in the art will be able to determine whether embodiments of the claimed invention are inoperative or operative without undue experimentation, because one of skill in the art can clearly determine whether the administration of R(-)-desmethylselegiline results in increased γ-interferon production.

d. The Predictability of the Art and the Amount of Experimentation Necessary Do Not Weigh Against Enablement

Finally, the Action states that "the practitioner would turn to trial and error experimentation in order to determine the 'conditions' caused by immune system dysfuntion [sic] (associated with gamma-IFN) in mammals that would respond to the claimed method of treatment (employing the claimed composition)." 5-17-05 Office

Action, p.5. But as clearly set forth in the specification, immune system dysfunction conditions associated with reduced levels of γ-interferon production are already well known to those of skill in the art, and any such conditions that did not respond to the claimed method of treatment by demonstrating an increase in γ-interferon production in the mammal, which could easily be identified by one of skill in the art, would not fall within the scope of the claim.

The specification sets forth that certain conditions produced by immune system dysfunction are associated with reduced levels of γ-interferon production. For example, AIDS and age-related immune system function loss are two representative examples of conditions associated with reduced levels of γ-interferon production. *See* Specification, p.8, ll.23-25. Experiments can be performed by a person of ordinary skill in the art to measure γ-interferon production in a mammal, and determine whether the level of production is less than standard values for the mammal. Such experiments would allow the person of ordinary skill in the art to identify conditions associated with reduced levels of γ-interferon production without any undue experimentation. Similarly, a person of ordinary skill in the art could easily identify conditions that did not respond to the claimed method of treatment as demonstrated by the absence of an increase in γ-interferon production. Such experimentation would be routine and not undue.

e. The Examiner Failed to Meet the Burden for Establishing a Proper Rejection Under §112

The MPEP dictates that "[a] specification disclosure which contains a teaching of the manner and process of ... using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be

taken as being in compliance with the enablement requirement of 35 U.S.C § 112, unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support." MPEP § 2164.04.

The Examiner has failed to establish that the scope of the disclosure and the scope of the claims are incongruent. Appellant's application describes the manner and process of using Appellant's claimed invention in terms which correspond to the claimed subject matter. The specification provides experimental data that supports Appellant's claims and shows that the administration of the R(-)-enantiomer of desmethylselegiline restores γ-interferon production. *See, e.g.,* Specification, pp.38-41. The specification also provides dosage ranges for the administration of the R(-)-enantiomer of desmethylselegiline when practicing Appellant's claimed invention. *See, e.g., Id.* at p.9, ll.8-21 & pp.41-42. Finally, Appellant's application describes conditions which may be treated through the administration of the R(-)-enantiomer of desmethylselegiline. *See, e.g., Id.* at p.8, ll.21-25.

The Examiner has not questioned the veracity of any of the Appellant's statements nor the validity of the experimental data contained in the application. The Examiner also has not offered any explanation of "why [she] doubts the truth or accuracy of any statement in [the] supporting disclosure," if she has any such doubts. MPEP § 2164.04. Instead, the Examiner has inappropriately focused on the underlying complexity of the immune system, rather than focusing on the claim language and the corresponding enabling disclosure.

Based on the foregoing arguments, Applicant respectfully asserts that the 35 U.S.C. § 112, first paragraph rejection is overcome. The Examiner has failed to show

that Appellant's claims are not commensurate in scope with Appellant's disclosure, or would require any undue experimentation by one of skill in the art. In addition, the Examiner has not questioned the objective truth of any of Appellant's enabling support for the claims. Therefore, the Examiner has not satisfied the burden for making an enablement rejection under the first paragraph of 35 U.S.C. § 112. Appellant therefore respectfully requests that the Board overturn the rejection.

B. The Obviousness Rejection Under 35 U.S.C. § 103 Should Be Overturned

1. Claims 26 and 34-62

The Examiner has rejected claims 26 and 34-62 as obvious over Borbe in view of Barton et al and Balsa et al. The Examiner argues that (1) Borbe teaches the oral administration of DMS to rats, which is an MAO-B inhibitor; (2) Barton associates immune dysfunction with conditions such as AIDS, Kaposi's sarcoma etc.; and (3) Balsa teaches that the activity of MAO-B is predominant in lymphocytes (L) and granulocytes (G). 5-17-05 Office Action, pp.5-6. The Examiner combines these references to conclude that "[o]ne of an ordinary skill in the art would have expected DMS, a monoamine oxidase inhibitor, to be effective in treating AIDS, tumors, cancers and other immune deficient conditions by inhibiting the action of MAO-B of immune cells i.e., lymphocytes and granulocytes." 5-17-05 Office Action, pp.6-7.

But as Appellant demonstrates below, the Examiner has failed to establish a *prima facie* case of obviousness in this rejection. First, the three references offer no teaching, suggestion, or motivation to combine their teachings to produce the claimed invention. Second, even if the references are combined (without any teaching,

suggestion, or motivation), the references do not teach or suggest *all* elements of the pending claims. This failure to teach all of the elements in turn means that one of skill in the art would have no reasonable expectation of success by combining the three references. Further, the references, even if combined, arguably teach away from the claimed invention. Indeed, the Examiner fails to explain in her combination of the above three references why one of skill in the art would expect that inhibiting MAO-B activity in lymphocytes and granulocytes will treat conditions produced by immune system dysfunction that is associated with reduced levels of γ-interferon production. Appellant certainly does not understand how the Examiner has come to such a conclusion based on the disclosures of the three combined references. Therefore, Appellant must conclude that the Examiner is using hindsight reconstruction to combine the three references together, which is impermissible.

a. The Examiner Failed to Establish a *Prima Facie* Case of Obviousness.

When setting forth an obviousness rejection, the MPEP clearly indicates that it is the Examiner who "bears the initial burden of factually supporting any *prima facie* conclusion of obviousness." MPEP § 2142. The MPEP sets forth that to establish a *prima facie* case of obviousness, three basic criteria must be met: (1) there must be a suggestion or motivation to combine the reference teachings either in the references themselves or in the general knowledge of one of ordinary skill in the art; (2) there must be a reasonable expectation of success; and (3) the references when combined must teach or suggest all the claim limitations. MPEP §§ 2142 & 2143. The Examiner bears the initial burden of factually supporting each of the three elements to establish a *prima facie*

case of obviousness. Appellant asserts that the Examiner has failed to establish any of the three criteria for such a *prima facie* case.

b. The Examiner Failed to Show a Motivation or Suggestion to Combine the References.

The motivation or suggestion to combine prong of the *prima facie* case for obviousness requires that the Examiner establish a suggestion or motivation to combine the reference teachings either within the references themselves, or within the knowledge of a person of ordinary skill in the art. MPEP § 2143.01. The Examiner has failed to present any suggestion or motivation to combine the reference teachings within the references themselves, or within the knowledge of one of skill in the art.

For example, Borbe is directed to the administration of R(-)-desmethylselegiline, an MAO-B inhibitor, to rats. The disclosure of Barton is completely unrelated to Borbe, in that there is no mention of R(-)-desmethylselegiline or MAO-B inhibitors. Barton instead studied neurological complications in patients with Kaposi's sarcoma, and found that opportunistic infections correlate with the degree of immune system dysfunction in a patient. The third reference cited by the Examiner, Balsa, is also unrelated to Borbe. While Balsa discloses that lymphocytes and granulocytes have MAO-B activity, there is no mention of R(-)-desmethylselegiline. Balsa also does not provide any teaching about the consequences of inhibiting the MAO-B activity of lymphocytes and granulocytes. Balsa is also unrelated to Barton. The mere knowledge that lymphocytes and granulocytes are part of the immune system hardly provides the motivation to combine these references. Therefore, as set forth above, the three references combined by the

Examiner offer no teaching, suggestion, or motivation to combine their teachings to produce the claimed invention.

Since the Examiner has not shown that the references themselves contain a suggestion or motivation to combine their teachings, she must present a "convincing line of reasoning as to why the artisan would have found the claimed invention to be obvious in light of the teachings of the references." MPEP § 2142 (citing *Ex Parte* Clapp, 227 USPQ 972, 973 (Bd. Pat. App. & Inter. 1985)). The Examiner has not provided any such convincing line of reasoning.

The Examiner argues that since "Balsa shows the activity of MAO-B in lymphocytes and granulocytes, the cell types that play a key role in immune system function and Barton teaches that immune deficiency is related to conditions such as cancer, neurological dysfunction, infection and AIDS....it would have been obvious for one of an ordinary skill in the art at the time of the instant invention to use DMS of Borbe for reducing the MAO-B activity in lymphocytes and granulocytes, which in turn play an important role in the development of immune deficient disorders such as Kaposi's' sarcoma, AIDS or other opportunistic infections because Barton associates immune dysfunction with conditions such as AIDS, Kaposi's' sarcoma etc., and Balsa teaches that the activity of MAO-B is predominant in G and L cells, which can be effectively inhibited by deprenyl." 5-17-05 Office Action, p.6.

The Federal Circuit has made it clear that a finding of a suggestion or motivation to combine must be 1) specific and 2) based on objective evidence. *See In re Sang-Su Lee*, 277 F.3d 1338, 1343 (Fed. Cir. 2002) (reviewing numerous cases discussing obviousness rejections). The Examiner has not made any specific factual findings to

support her statement that the combination she sets forth above would have been obvious to one of skill in the art. Likewise, the Examiner has not discussed any objective evidence that supports a finding of a suggestion or motivation to combine within the knowledge of one of skill in the art. Instead, the Examiner has engaged in hindsight reconstruction and impermissibly combined three references together without any clear explanation of the rationale or logic of the combination.

Since the Examiner has not provided a specific and objective factual discussion supporting her finding of a suggestion or motivation to combine the references in the knowledge of one of skill in the art, nor shown such a suggestion or motivation in the references themselves, the Examiner has failed to meet the first criterion for establishing a *prima facie* case of obviousness.

3. The Combination of References Does Not Teach or Suggest All Claim Limitations

The Examiner bears the initial burden as an element of a *prima facie* case of obviousness of factually establishing that the references, when combined, teach or suggest *all* of the claim limitations. MPEP § 2142. The references combined by the Examiner, however, fail to teach all of the claim limitations of the pending claims.

As the Examiner admits, the references do not explicitly discuss a reduction in the levels of γ-interferon production, which is an element of each of the pending claims, or that the administration of R(-)-desmethylselegiline leads to an increase in γ-interferon production. 5-17-5 Office Action, p.7. Instead, the Examiner argues that "absent showing the evidence to the contrary, it is the position of the examiner that the claimed composition implicitly restores the levels of gamma-IFN." *Id.* But, the Examiner

presents no evidence that one of skill in the art would have understood this from the cited combination of references.

Appellant assumes that the above rejection made by the Examiner is an inherency argument. *See* MPEP § 2112. The MPEP is clear that when an examiner relies on a theory of inherency, "the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art." MPEP § 2112, Part III (*citing Ex parte Levy*, 17 USPQ.2d 1461, 1464 (Bd. Pat. App. & Inter. 1990)). The Examiner has provided no such basis in fact or technical reasoning to support her rejection. Instead, she attempts to shift the burden to Appellant, which is impermissible, by making the conclusory statement that "absent showing the evidence to the contrary, it is the position of the examiner that the claimed composition implicitly restores the levels of gamma-IFN." 5-17-05 Office Action, p.7.

Since the Examiner has not met her burden of establishing a *prima facie* case of obviousness, Appellant is under no obligation to submit evidence that the combined references do not implicitly teach the restoration of γ-interferon production. Without any evidence to support her rejection, Appellant need not present evidence to refute this conclusory statement made by the Examiner. Therefore, once again, the Examiner has failed to meet another criterion for establishing a *prima facie* case of obviousness.

4. The Examiner Failed to Show a Reasonable Likelihood of Success.

As stated previously, the Examiner bears the initial burden of factually establishing a reasonable expectation of success when combining the teachings of the references as an element of a *prima facie* case of obviousness. MPEP § 2142. As

Appellant has already established, there is no motivation or suggestion to combine the cited references, and even if combined, the references do not disclose every element of the pending claims. Therefore, there can be no expectation of success. Furthermore, even if one of skill in the art were to combine the cited references, he or she would not arrive at the claimed invention because the references, in fact, teach away from the claimed invention, and thus cannot render the claims obvious.

The Examiner states that "[o]ne of ordinary skill in the art would have expected DMS, a monoamine oxidase inhibitor, to be effective in treating AIDS, tumors, cancers and other immune deficient conditions by inhibiting the action of MAO-B of immune cells i.e. lymphocytes and granulocytes." 5-17-05 Office Action, p.6. The Examiner provided no other discussion of the likelihood of success for combining the cited references.

The Examiner's statement that one of skill "would have expected DMS... to be effective" cannot satisfy the Examiner's burden. The Examiner has offered no factual evidence in support of a reasonable expectation of success in combining the references. Without such support the Examiner's broad sweeping statement is insufficient to meet the factual burden established by MPEP §2142 for showing a reasonable expectation of success. Since the Examiner has not factually established a reasonable expectation of success in utilizing R(-)-desmethylselegiline to treat a condition produced by immune system dysfunction, she has failed to establish a *prima facie* case of obviousness.

As stated above, even if a person of skill in the art were able to combine the cited references, the references arguably teach this person away from the claimed invention. For example, Balsa discloses that lymphocytes and granulocytes in pig blood have

MAO-B activity. There is no indication that the cells studied in Balsa are mutant or otherwise abnormal. Therefore, one of skill in the art would conclude that lymphocytes and granulocytes, when properly functioning in the immune system, have MAO-B activity. Why then would one of skill in the art conclude that inhibiting the natural MAO-B activity of lymphocytes and granulocytes would have beneficial results for an organism? Certainly, one of skill in the art would reasonably expect that interfering with the normal activity of immune cells would be more likely to have a bad result than a good result. Since Balsa does not disclose what effect inhibiting the MAO-B activity of lymphocytes and granulocytes would have on the immune system, there would be no way for one of skill in the art to answer this question. Therefore, the Examiner's use of hindsight reconstruction to supplement the disclosures of these references is undeniable.

In light of the above arguments showing the Examiner's failure to show a suggestion or motivation to combine the references, that the combined references teach or suggest all of the claim limitations, and a reasonable expectation of success for such a combination, Appellant asserts that the Examiner has failed to establish a *prima facie* case of obviousness and respectfully requests that the Board overturn the Examiner's rejection under 35 U.S.C. § 103(a).

C. Conclusion

Appellant respectfully submits that based on the foregoing observations and arguments, all pending claims are enabled and patentable. It is therefore respectfully requested that the Board overturn the Examiner's rejections.

Respectfully submitted,

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Date: March 16, 2006

VIII. CLAIMS APPENDIX

Claims 1-25 (Canceled).

Claim 26. (Previously presented) A method of treating a condition in a mammal produced by immune system dysfunction that is associated with reduced levels of γ-interferon production, which comprises administering to the mammal the R(-) enantiomer of desmethylselegiline, or a pharmaceutically acceptable acid addition salt thereof, at a daily dose, administered in a single or multiple dosage regimen, of at least about 0.015 mg, calculated on the basis of the free secondary amine, per kg of the mammal's body weight, wherein the administration of the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof leads to an increase in γ-interferon production in the mammal.

Claims 27-33 (Canceled).

Claim 34. (Previously presented) The method of claim 26, wherein said R(-) enantiomer of desmethylselegiline is in a substantially enantiomerically pure state.

Claim 35. (Previously presented) The method of claim 26, wherein the condition produced by immune system dysfunction is caused by infectious disease.

Claim 36. (Previously presented) The method of claim 26, wherein the immune system dysfunction is age-dependent.

Claim 37. (Previously presented) The method of claim 26, wherein the condition produced by immune system dysfunction is AIDS.

Claim 38. (Previously presented) The method of claim 26, wherein the condition produced by immune system dysfunction is cancer.

Claim 39. (Previously presented) The method of claim 26, wherein the condition produced by immune system dysfunction is in response to a vaccine.

Claim 40. (Previously presented) The method of claim 26, wherein the daily dose is between about 0.5 mg/kg and about 1.0 mg/kg.

Claim 41. (Previously presented) The method of claim 26, wherein the daily dose is at least about 1.0 mg/kg.

Claim 42. (Previously presented) The method of claim 26, wherein the mammal is a human.

Claim 43. (Previously presented) A method of treating a condition in a mammal produced by immune system dysfunction caused by cancer chemotherapy which is associated with reduced levels of γ-interferon production, which comprises administering to the mammal the R(-) enantiomer of desmethylselegiline, or a pharmaceutically acceptable acid addition salt thereof, at a daily dose, administered in a single or multiple dosage regimen, of at least about 0.015 mg, calculated on the basis of the free secondary amine, per kg of the mammal's body weight, wherein the administration of the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof leads to an increase in γ-interferon production in the mammal.

Claim 44. (Previously presented) The method of claim 43, wherein the R(-) enantiomer of desmethylselegiline is in a substantially enantiomerically pure state.

Claim 45. (Previously presented) The method of claim 43, wherein the mammal is a human.

Claim 46. (Previously presented) A method of treating a condition in a mammal produced by immune system dysfunction that is associated with reduced levels of γ-interferon production, which comprises administering to the mammal the R(-) enantiomer of desmethylselegiline, or a pharmaceutically acceptable acid addition salt thereof, wherein the administration of the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof leads to an increase in γ-interferon production in the mammal.

Claim 47. (Previously presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline is in a substantially enantiomerically pure state.

Claim 48. (Previously presented) The method of claim 46, wherein the mammal is a human.

Claim 49. (Previously presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof is administered orally.

Claim 50. (Previously presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof is administered non-orally.

Claim 51. (Previously presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof is administered parenterally.

Claim 52. (Previously presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof is administered transdermally.

Claim 53. (Previously presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof is administered buccally or sublingually.

Claim 54. (Previously presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof is administered intravenously.

Claim 55. (Previously presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof is administered subcutaneously.

Claim 56. (Previously presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline or a pharmaceutically acceptable acid addition salt thereof is administered intra-peritoneally.

Claim 57. (Previously presented) The method of claim 46, wherein the R(-) enantiomer of desmethylselegiline is administered at a daily dose of at least about 0.015 mg/kg of the mammal's body weight, calculated on the basis of the free secondary amine.

Claim 58. (Previously presented) The method of claim 46, wherein the condition produced by immune system dysfunction is caused by infectious disease.

Claim 59. (Previously presented) The method of claim 46, wherein the immune system dysfunction is age-dependent.

Claim 60. (Previously presented) The method of claim 46, wherein the condition produced by immune system dysfunction is AIDS.

Claim 61. (Previously presented) The method of claim 46, wherein the condition produced by immune system dysfunction is cancer.

Claim 62. (Previously presented) The method of claim 46, wherein the condition produced by immune system dysfunction is in response to a vaccine.

IX. EVIDENCE APPENDIX

The following reference was cited in Appellant's Response to the Office Action dated October 21, 2004, filed January 21, 2005.

Exhibit 1. Billiau, A., *Interferon-γ: Biology and Role in Pathogenesis*, ADV. IMMUNOL. 62:61-130 (1996)

X. RELATED PROCEEDINDS APPENDIX

No decisions have been rendered in the related appeals described in Section II.

Exhibit 1

ADVANCES IN IMMUNOLOGY, VOL. 62

Interferon-γ: Biology and Role in Pathogenesis

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I. Introduction

The protein that is now called interferon-γ (IFN-γ) was discovered independently by two groups of investigators and was originally given two different names after the biological activities detected: (a) type II or immune interferon, and (b) macrophage-activating factor (MAF). Homonymy of IFN-γ with IFN-α and -β does not imply molecular relationship but merely reflects sharing of the biological property to protect cells against virus infection. The corresponding bioassay is the so-called antiviral assay, which consists in demonstrating that cultured cells exposed to IFN-γ resist destruction by a standard challenge virus. Historically, the name interferon refers to the phenomenon of interference, i.e., the fact that cells infected with any one virus species are relatively resistant to infection with viruses of other species. However, whereas IFN-α and -β (collectively called type I interferons) do play a role in such interference, IFN-γ is not involved.

The earliest report dealing with IFN-γ is probably that by Wheelock in 1965 (1), demonstrating that an interferon-like antiviral activity appears in supernatants of mononuclear cells exposed to a mitogen. In the early seventies, the terms "type II interferon" (2) and "immune interferon" (3) were coined. The term immune interferon remained in use for some time in recognition of the awareness that the activity is associated with a protein physicochemically distinct from that responsible for the then-classical interferon, that production of the factor is the prerogative of immune competent cell types, and that the factor possesses immune regulatory properties distinct from those of classical interferon. In 1980, an international interferon nomenclature committee (4) agreed on a new name for all interferons. Type I interferons, then known as leukocyte and fibroblast interferons, were renamed IFN-α and -β, respectively; immune interferon was renamed IFN-γ.

Availability of pure preparations of IFN-γ and of monospecific antibodies made it possible to prove that MAF activity in biological fluids is largely, if not entirely, accounted for by IFN-γ. The term MAF appeared in the literature around 1980; it refers to the ability of supernatants of mitogen- or antigen-challenged mononuclear cell cultures to augment various biological activities of macrophages. That stimulated lymphocytes' supernatants have these macrophage-stimulating abilities was first re-

ported in 1966 by Bloom and Bennett (5a). MAF bioassays have variably relied on intracellular killing of parasites or increased oxidative metabolism (5), enhanced expression of class II antigens (6), or enhanced tumor cell killing (7). Characterization of MAF with monoclonal antibodies soon revealed its identity with IFN-γ (5). It is possible that other cytokines besides IFN-γ have MAF-like activities, but they seem to be of less importance.

Ever since their discovery, interferons have attracted vivid interest from clinical investigators. Medical interest in IFN-γ stems from awareness that a prominent target cell of IFN-γ, the macrophage, occupies a central position in the immune system. Adequate function of the IFN-γ/macrophage system is essential for natural as well as acquired resistance to infection and cancer. Malfunctioning of the system is recognized to be instrumental in inflammatory and autoimmune disease. Not surprisingly, therefore, of all known cytokines, IFN-γ belongs to the small group that has already been tested for therapeutical effects in patients. Being among the first cytokines to be discovered, IFN-γ is the subject of an extensive and still expanding literature. Surprisingly, only a few reviews have been devoted to IFN-γ (8, 9). Here, the salient issues and most recent advances concerning IFN-γ's biological function are reviewed so as to provide a framework for understanding its role in disease.

II. Structure and Structure–Function Relationship

IFN-γ is a glycoprotein the size, amino acid sequence, and glycosylation of which are well conserved among animal species. Most studies on structure have been done on either human or mouse IFN-γ. In its biologically active form, IFN-γ is a 34-kDa homodimer stabilized by noncovalent forces. The peptide is N-glycosylated on two sites. Natural IFN-γ is heterogeneous in size and charge due to enzymatic trimming of the carboxy terminus and to variation in degree of glycosylation. This heterogeneity seems to be unimportant for biological activity on cells but might well influence dynamics of tissue distribution.

X-ray crystallographic analysis (10) has revealed that the subunits consist of six α-helices, accounting for 62% of the molecule, with no β-sheet domains. The two subunits, each of which is elongated in shape, are held together in an antiparallel configuration by intertwining of the helical domains. The amino terminus of each chain is juxtaposed to the carboxy terminus of the opposing chain. The symmetry in this structure allows for IFN-γ to bind a pair of identical receptor peptides, as has been demonstrated by analysis of the crystal structure of an IFN-γ/IFN-γR complex (11). Peptide stretches at both the amino and the carboxy termini of

IFN-γ seem to have binding properties for the receptor, as demonstrated by competition-type experiments using the corresponding peptides (12). The amino-terminal binding region has been identified (13).

By substituting glutamine residues for asparagine residues at positions 25 and/or 97, four differently glycosylated mutant forms of human interferon could be obtained (14) and studied. These replacements were found to affect both production yields by insect cells as well as correct dimer formation. However, specific biological activity of the correctly formed dimers remained largely unaffected. Studies employing monoclonal antibodies and recombinant mouse/man hybrid IFN-γ molecules have provided suggestive but not conclusive evidence that mouse IFN-γ is composed of distinct functional domains that induce different biological responses. A carboxy-terminal domain was proposed to be sufficient for macrophage activation, whereas both a carboxy- and a different amino-terminal domain would be required for induction of the antiviral effect in cells (15).

Neutralizing monoclonal antibodies against human IFN-γ fall into four epitope-specific groups (E1, E2, E1/E2 overlap, and E3) identifying three epitopes (E1, E2, and E3), each of which is somehow involved in biological activity (16). However, none of these epitopes identifies the receptor-binding site. Therefore, it has been hypothesized that receptor binding and signal transduction functions are separate on the IFN-γ molecule and that the neutralization epitopes so far identified represent signal transduction functional domains. Only epitope E1 has to date been identified with a particular stretch of amino acids (residues 84–94). Intriguingly, the amino acid sequence at this domain resembles the nuclear localization (targeting) signal (NLS) of many nuclear proteins.

III. Producers and Production of IFN-γ

IFN-γ is a typical lymphokine, being produced exclusively by NK cells and certain subpopulations of T lymphocytes, namely the TH1 subclass of $CD4^+$ lymphocytes and certain $CD8^+$ lymphocytes (17) (Fig. 1). In the human system, T cells that express the activation-dependent CD30 membrane antigen have been identified as the principal subset producing IFN-γ (18). A single literature report describes production of IFN-γ by mononuclear phagocytes (19).

To produce IFN-γ, lymphocytes need to be activated, as a result of which they also secrete other cytokines, e.g., IL-2 in the case of TH1 cells. As a general rule, production of IFN-γ by either NK or T cells requires cooperation of accessory cells, mostly mononuclear phagocytes, which also need to be in some state of activation. One aspect of the ancillary role of

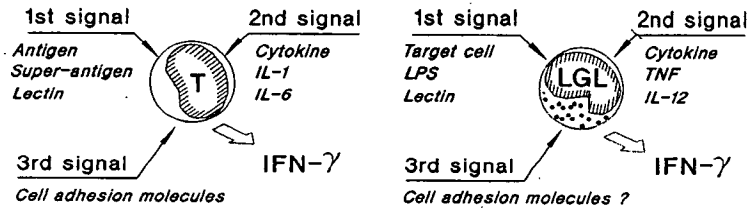

FIG. 1. Producers of IFN-γ. IFN-γ is produced by only two types of cells: T lymphocytes (both TH1 and $CD8^+$ cells) and large granular lymphocytes (NK cells). Constitutive production is minimal or nonexistant. Optimally induced production occurs when the cells receive a combination of three types of signals: (a) a specific or aspecific ligand for the receptor, (b) a balanced assembly of cytokines, and (c) contact with accessory cells through cell adhesion molecules.

these cells is that they need to produce regulatory cytokines, e.g., TNF-α, IL-12, or IFN-β, but also that they need to make contact with the lymphocytes through intercellular adhesion molecules. The evidence that has led to this more or less generally applicable schedule is diverse, as is summarized below.

Gram-positive bacterial components, e.g., *S. aureus* preparations, induce NK cells to produce IFN-γ *in vitro* (20). In gram-positive bacterial infections, e.g., in listeriosis, NK cells contribute a large part in IFN-γ production, which accounts for resistance of the host to the infection (21). Similarly, in parasitic infections, e.g., in *Leishmaniasis* models, early production of IFN-γ, which is of crucial importance for the further course of the infection, is accounted for by NK cells (22). It should be noted, however, that for exogenous stimuli to induce IFN-γ production in NK cells, the presence of cytokines produced by monocytes is mostly required. Thus, in *scid* mice, induction by *Listeria* of IFN-γ production by NK cells requires monocytes. The role of the monocyte could be to produce TNF. TNF can indeed substitute for monocytes (23, 24) and TNF-receptor knockout mice are highly susceptible to *Listeria* infection (25), but an additional monocyte-produced cytokine seems to be required for optimal production. This is most likely IL-12, known to strongly upregulate IFN-γ production by NK cells (26).

A comparison of IFN-γ production by murine splenocytes of responsive and nonresponsive strains led to the conclusion that, for gram-negative bacteria to induce IFN-γ, the presence of mononuclear phagocytes and production of IFN-β is required (27). IFN-γ produced in response to injection of endotoxins of gram-negative bacteria apparently originates from NK cells as well as $CD4^+$ and $CD8^+$ T cells, because the mRNA is detectable in all three populations (28). Again, in endotoxin-injected mice,

release of IL-12 into the circulation precedes that of IFN-γ, and pretreatment with anti-IL-12 antibody inhibits such production of IFN-γ (28). Nevertheless, in the lethal Shwartzman reaction caused by two consecutive injections of endotoxin, elimination of NK cells, but not of $CD4^+$ or $CD8^+$ T cells, prevents the toxic manifestations of the reaction (29). However, not only monocyte-derived cytokines, but also IL-2 produced by TH1 cells can stimulate IFN-γ production by NK cells (22).

As an exception to the general rule that IFN-γ induction in NK cells requires help from accessory cells, the superantigen SEB reportedly can independently induce IFN-γ production in human NK cells (30); activation into cytotoxic cells, however, was found to require the presence of cytokines delivered by T cells.

The situation may be quite different in mycobacterial infections (31). IL-12 induction by *Mycobacterium bovis* (BCG) in murine bone marrow-derived macrophages was found to depend on the presence of IFN-γ and TNF, as it failed to occur in macrophages of mice lacking functional receptors for either cytokine or was prevented by addition of neutralizing antibodies to either cytokine. Also, *in vivo*, IL-12 production in the spleen was absent in TNF-α or IFN-γ receptor knockout mice. Therefore, in this system IL-12 does not precede IFN-γ as seems to be the case in other systems.

In mice infected with *Toxoplasma*, both $CD4^+$ T cells and NK cells contribute to the early IFN-γ response. Monoclonal antibodies against IL-12 were found to reduce both IFN-γ production and resistance to the infection (32).

IFN-γ production by T lymphocytes occurs when these cells are activated by antigens, e.g., a viral antigen presented in MHC class I molecule context, to the idiotypic receptor (33). Activation by antigens presented by professional antigen-presenting cells also involves cell adhesion molecules and their corresponding ligands, Thus, enhancement of IFN-γ production by signaling through the CD2/LFA-3 pathway has been demonstrated in studies with CD2-blocking antibodies and with cells transfected with LFA-3 (34). Moreover, cytokines and other mediators released by the antigen-presenting cells, as well as by the lymphocytes themselves and by bystander lymphocytes, have a profound regulatory effect on the quantities of IFN-γ that are released. IL-2 is classically said to stimulate production of IFN-γ. In allergen-driven murine T cells, IFN-γ production is strictly dependent on prior production of IL-2, whereas IL-4 production is IL-2 independent (35). IL-10, on the other hand, inhibits IFN-γ production (see below).

Crucial to the production of IFN-γ during antigen-specific immune responses *in vivo* is the development of naive $CD4^+$ T cells into either TH1 or TH2 lymphocyte clones. Total production of IFN-γ during later

phases of immune responses is more elevated if the antigen-specific TH1 cells predominate. Using T cell receptor transgenic CD4+ T cells, it has been shown that IL-12 substantially enhances development into cells that produce IFN-γ on restimulation; IL-4, by contrast, inhibits such development (36). The stimulatory effect of IL-12 on IFN-γ production by activated human T cells is largely mediated by its effect on the CD30-expressing subset (37).

Production of IFN-γ by T cells is also under regulatory control of prostaglandins. PGE2 inhibits production by TH1 cells of IFN-γ and IL-2 but not production of IL-4 by TH2 cells. cAMP is involved in this control mechanism, which allows PGE2 to skew immune responses in the TH2 direction (38).

Some information is available on the molecular basis of IFN-γ induction. Analysis of the promoter region has yielded evidence for the existence of three distinct response elements upstream of the TATA box. One of these (BE), originally detected by its silencer activity, was shown to be able to interact with two protein complexes, S and E, with silencer and enhancer activity, respectively. The S protein complex recognizes a sequence similar to that recognized by nuclear factor AP2, but does not contain AP2 itself. The E complex contains nuclear factor YY1 (39).

IV. Biochemical Basis of IFN-γ Action

A. The Receptor

The IFN-γ receptor complex on human mononuclear leukocytes consists of at least three distinct proteins (40). One of these, the IFN-γ receptor α-chain (IFN-γRα), is the membrane protein that primarily binds IFN-γ with high affinity and the gene of which is located on human chromosome 6 (41) or mouse chromosome 10. It encompasses three domains (42, 43): the extracellular ligand-binding, the transmembrane, and the intracellular domains. When the human or mouse variants of this protein are expressed in heterologous cells, they do bind the homologous IFN-γ but fail to transmit any signal to the cell. Chimeric mouse/human constructs of the protein, expressed in cells of either species, has indicated that, for signals to be transmitted, the extracellular domain must interact with one or more species-matching proteins (44). Studies with cell hybrids have revealed one of these proteins to be coded for by human chromosome 21 (45) or mouse chromosome 16 (46). Both in murine and in human cells, the species-matching protein has been fully characterized by molecular cloning (47, 48). This protein, termed IFN-γRβ, has a structure resembling that of IFN-γRα. Interaction of the α-chain extracellular domain with this protein is sufficient for transmission of the signal to express MHC class

II molecules, but insufficient for expression of the antiviral state. Therefore, interaction with a third protein is surmised. The gene for this third factor is also located on human chromosome 21 (49).

IFN-γ causes dimerization of recombinant soluble receptor (50). Analysis of the crystal structure of the complex revealed that the two IFN-γRα chains in this complex remain separated from each other (11).

Studies aimed at locating the extracellular IFN-γRα domains responsible for interaction with ligand and with the β-chain have been performed by replacing segments of the α-chain in human receptor with corresponding murine counterparts (51). This has led to a provisional model in which both the species-specific N termini and the conserved C termini of the IFN-γ homodimer bind to the extracellular domains of an α-chain receptor pair. The model also predicts that multiple segments of the extracellular α-chain cooperate to bind with β-chain. Another approach to elucidate interaction of IFN-γ with its receptor has consisted of studying binding of α-chain peptide fragments with IFN-γ (52). These studies have led to the suggestion that IFN-γ recognizes a binding site in the cytoplasmic domain of the α-chain.

Aside from the ubiquitous high-affinity IFN-γ-binding protein, an additional distinct low-affinity receptor has been reported to occur on cells of the mononuclear phagocyte lineage (53).

Expression of IFN-γ-binding sites on cells of the monocytic lineage is regulated by other cytokines. A twofold increase was recorded after exposure to TNF-α or IL-1 (54). Such upregulation was found to be associated with increased IFN-γ-induced expression of MHC class II Ag (54). Upregulated transcription of the IFN-γ receptor gene has also been reported in a human carcinoma cell line treated with TNF (55) and in a monocytic cell line treated with TNF-α or IL-6 (56). Hormonal control of IFN-γR expression is evidenced by studies on mRNA levels in maternal uterine and embryonic hematopoietic cells during gestation in mice (57). The distribution of IFN-γR mRNA among leukocytes in cycling uteri was found to be low and fairly uniform among the cell populations, with macrophages exhibiting a maximal expression level in diestrous uteri. In pregnant uteri, hematopoietic cells were found to proliferate and express IFN-γR mRNA to degrees that varied among different cell types; concomitantly, these cells expressed activation markers.

B. Signal Transduction, Transcription Factors, and Response Elements (Fig. 2)

Binding of IFN-γ to the membrane receptor complex transmits signals to the cytoplasma and nucleus by the Jak–STAT mechanism of signal transduction (58). This mechanism involves a cascade of tyrosine phosphor-

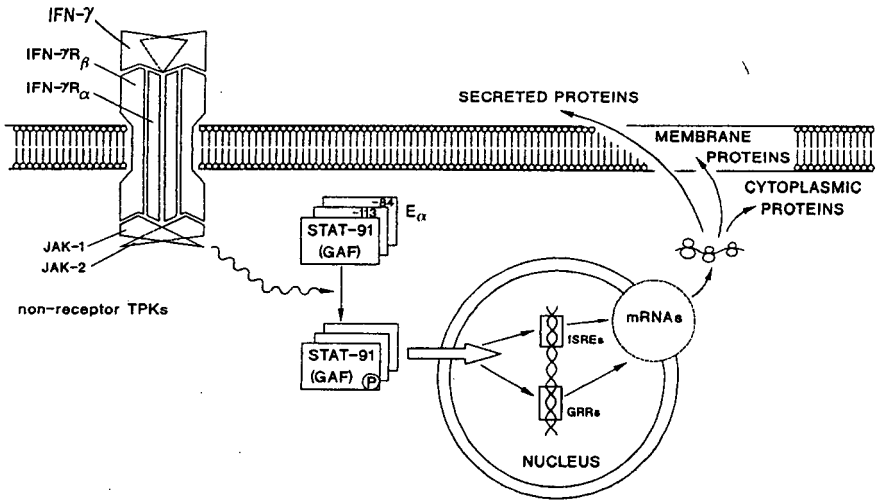

FIG. 2. Schematic model for ligand–receptor interaction and signal transduction in IFN-γ-responding cells. The IFN-γ homodimer binds to the IFN-γRα chain which, as a result of this binding, also dimerizes and associates to two molecules of the IFN-γRβ chain. At the intracellular side, the IFN-γRα/IFN-γRβ chain complex associates with the nonreceptor tyrosine phosphokinases JAK-1 and JAK-2, thereby triggering their (auto)phosphorylation. JAK-2 catalyzes phosphorylation of STAT-1 [formerly STAT-91, 91 kDa Eα subunit, or gamma-activator factor (GAF)]. STAT-1 translocates to the nucleus where it binds to interferon-sensitive response elements (ISRE) and gamma-responsive elements (GRR) in the promotor regions of several IFN-γ-responsive genes. Derepression of these genes leads to formation of mRNAs and proteins, some of which are located intracellularly (e.g., 2′,5′-oligoadenyl synthase), others in the membrane (e.g., class II MHC gene products), and still others are secreted extracellularly (e.g., IFN-γ-induced cytokines).

ylations on cytoplasmic proteins, the so-called STAT (for *s*ignal *t*ransducer and *a*ctivator of *t*ranscription) proteins. The STATs were discovered in the context of investigations aimed at elucidating the mode of action of interferons but were later found to play roles in signal transduction of many other cytokines and growth factors (59–62). In essence, the intracellular part of the IFN-γ receptor protein does not have catalytic activity but interacts with nonreceptor-type tyrosin protein kinases belonging to the Jak (for Janus kinase) family. These kinases were originally discovered by low stringency hybridization with probes to domains of known protein kinases and by PCR with primers based on highly conserved sequences of known protein tyrosine kinases. Three such Jaks (TYK-2, JAK-1, and JAK-2) have so far been implicated in receptor triggering by IFNs. JAK-2 is required for cells to respond to IFN-γ but not to IFN-α/β (63). JAK-1 seems to be associated with the IFN-γ receptor before ligand

binding, whereas JAK-2 is recruited into the ligand–receptor complex within seconds after IFN-γ is added to cells (64). The mechanism of phosphorylation of and by Jaks has not completely been defined. A working hypothesis is that Jaks, by binding to the dimerized receptor complex, acquire the ability to phosphorylate each other and the receptor peptides. This phosphorylated receptor/Jak complex may next constitute the catalyst for phosphorylation of the STATs. Tyr_{440} on one of the IFN-γ receptor chains is essential for activation by IFN-γ and a phosphopeptide fragment containing Tyr_{440} binds to STAT-1 (65). The Jaks seem to lack an src homology 2 (SH2) domain (i.e., a phophotyrosine-binding domain), a characteristic of other nonreceptor tyrosine kinases and their substrates. STAT proteins do contain SH2 domains. Experiments in which the SH2 domains between STAT-1 and STAT-2 were swapped demonstrated that these domains specifically direct selection of a particular STAT for phosphorylation at the IFN-γ receptor as well as subsequent dimer formation between STAT proteins (66).

Members of the STAT family vary in molecular mass from 84 to 113. They possess SH2 and SH3 domains. Activation requires phosphorylation on specific tyrosines followed by formation of dimers or multimers that are translocated to the nucleus where they bind to specific promotor regions (response elements) and act as transcription factors. The system of STATs and response elements involved in IFN-γ action resemble that involved in IFN-α/β action (67). The IFN-α/β-connected transcription factors encompass ones that are inducible [E (ISGF3) and M (ISGF2 = IRF-1)] and one that is constitutive [C (ISGF1)]. These interferon-stimulated gene factors are composed of subunits, e.g., E is composed of Eα and Eγ. Eγ (STAT48) is the actual DNA-binding protein, requiring one or more of the other STATs to be translocated to the nucleus. Eα is itself composite, containing three peptides (STAT84 or STAT-1α, STAT91 or STAT-1β, and STAT113 or STAT-2). Following interaction of IFN-α with the receptor complex, the three components of Eα are phosphorylated and subsequently translocated to the nucleus. In response to IFN-γ, only STAT91 [formerly called gamma-activated factor (GAF)] is phosphorylated on tyrosine and translocated. Thus, the two types of interferons trigger phophorylation of different combinations of the same latent complex of cytoplasmic transcription factors, which then interact with different sets of genes (68, 69).

Depending on whether the transcription factor complex activated by IFN-α or -β contains the DNA-binding elements IRF-1 or IRF-2 (interferon-regulatory factors), it stimulates or inhibits transcription of those genes that contain interferon-sensitive response elements (ISREs). IRF-1 and IRF-2 are DNA-binding factors originally identified as regulators of the IFN-α and IFN-β genes (70, 71). They are structurally related,

particularly in their amino-terminal regions which confer specificity for DNA binding. Both factors bind to the same region in the promoters of the IFN-α and -β genes, but also to ISREs in the promoters of several IFN-inducible genes. Thus, IRF-1 and -2 are involved in regulating the induction as well as the action of IFN-α and -β.

IRF-1 functions as a transcription activator, whereas IRF-2 suppresses the action of IRF-1. In the context of cell growth regulation, IRF-1 has growth-inhibitory whereas IRF-2 has growth-promoting activity. The genes for IRF-1 and IRF-2 themselves are inducible by infection of the cells with viruses or exposure to cytokines (IFNs, TNF-α, IL-1, IL-6, or LIF) or prolactin.

Some IFN-γ-responsive genes contain ISREs; it seems that, in these genes, the presence of ISRE is sufficient for responsiveness. Other genes known to be triggered by IFN-γ, e.g., class II MHC and Fc-receptor genes, do not contain ISREs, but do contain other IFN-γ-specific response sequences, e.g., the gamma response region (GRR) in the promotor of FcγRI (72), the γRE-1 element in the promoter of the *mig* gene (*mig* is the monokine induced by IFN-γ, a member of the interleukin-8 family) (73) and the γ-activated sequence (GAS) in the IRF-1 gene (71).

Signaling systems other than the ISGFs may be involved in IFN-γ action. Many studies have indeed suggested involvement of PKC and/or Ca^{2+} calmodulin in the action of IFN-γ. For instance, induction of MHC antigen expression by IFN-γ was found to be associated with rapid increases in PKC activity (74) and to be blocked by H7, an inhibitor of PKC (75). Furthermore, activation of PKC following exposure to interferon was found to require increased $[Ca^{2+}]_i$ and activation of the calmodulin system as well as increased PI breakdown and tyrosine phosphorylation (76). Insofar as PKC is involved, tyrosine phosphorylation is probably a proximal event (77).

There is speculation that the IFN-γ protein, in addition to using the membrane receptor-activated phosphorylation cascade, also interacts with an intracellular or even intranuclear receptor. Binding of IFN-γ to its receptor is followed by endocytosis of the ligand/receptor complex (78, 79). Inhibition of receptor-mediated endocytosis, e.g., by acidification of the medium, inhibits induction of MHC antigen induction by IFN-γ, indicating that internalization of the ligand/receptor complex contributes to IFN-γ activity (80). Studies with murine macrophages have confirmed uptake of the receptor/ligand complex and have also indicated that the receptor is neither up- nor downregulated by ligand binding, but is being recycled (81). Several observations have given ground for speculation that transportation of IFN-γ from the cell membrane to some nuclear receptor system contributes to the establishment of biological effects of IFN-γ on

cells. Such observations include demonstration of biological activity of human IFN-γ in murine cells following microinjection (82) or intracellular production (83). Similar results were obtained in studies involving treatment of macrophages with liposome-encapsulated IFN-γ (84). Accumulation of IFN-γ in the cell nucleus, as demonstrated by autoradiography and direct immunofluorescence, gives further credibility to this concept (85). Also, the IFN-γ protein contains two conserved stretches of basic amino acids that resemble the NLS of large T antigen of simian virus 40 (86). These stretches are exposed on the surface of the IFN-γ molecule. Antibodies directed against the amino-terminal stretch were found to neutralize biological activity of IFN-γ without preventing its binding to the membrane receptor (86). Removal of the C-terminal NLS-like stretch was likewise found to result in abolishment of biological activity (87).

C. Biochemical Changes in Cells

1. Tryptophan Metabolism

One of the proteins induced by IFN-γ is the enzyme indoleamine-2,3-dioxygenase (IDO). IDO is the first enzyme of the kynurenine pathway that links tryptophan to alanine and acetyl CoA. It catalyzes the oxidative cleavage of the pyrrole ring in tryptophan to yield *N*-formyl-kynurenine. Kynurenine is transformed by enzymes in liver and brain to alanine and metabolites, some of which, such as quinolinic acid, have neuroactive or toxic potential.

Induction of IDO by IFN-γ explains why exposure to endogenous or exogenous IFN-γ is often associated with decreased serum tryptophan levels and increased levels of kynurenine in serum and urine. It has been argued that quinolinic acid, released as a result of exposure to endogenous IFN-γ, may be responsible for central nervous system manifestations in AIDS patients, in patients receiving cytokine therapy, or in patients with so-called cytokine release syndromes, such as the first dose reaction in renal allograft recipients given anti-CD3 antibody as the immunosuppressant (88, 89).

Induction of IDO by IFN-γ may indirectly function as a scavenger mechanism of the superoxide anion because this anion is used in the IDO-catalyzed conversion of tryptophan to *N*-formyl-kynurenine. The cell- and tissue-damaging effect of IFN-γ may thereby be dampened.

IFN-γ-induced depletion of tryptophan has also been speculated to contribute to te antiproliferative effect of IFN-γ. In a panel of cultured human ovarian tumor cell lines, induction of IDO could not be correlated with the sensitivity of the lines to the antiproliferative effect of IFN-γ (90). However, IFN-γ was found to also induce the synthesis of trypto-

phanyl tRNA synthetase, which might in resistant cell lines blunt the consequences of tryptophan depletion for overall protein synthesis.

2. *Generation of Reactive Oxygen*

IFN-γ is well known to potentiate respiratory burst responsiveness of macrophages to stimulants, resulting in increased production of highly reactive oxidants such as H_2O_2 and the superoxide anion (O_2^-) (5). These effects of IFN-γ are believed to be due to regulation of the transcription of genes coding for enzymes of the NADPH oxidase system (91). This membrane-associated system is dormant in resting cells, but becomes activated during phagocytosis or on interaction with certain soluble stimuli. In addition, IFN-γ also stimulates production of nitrogen monoxide (NO), which in turn may react with H_2O_2 to generate reactive oxygen.

3. *Pteridin Metabolism: Generation of Tetrahydrobiopterin*

Tetrahydrobiopterin is a limiting factor in the synthesis of NO (see below). Pteridins are synthesized from GTP, the first step being conversion to 7,8-dihydroneopterin-3′-triphosphate by the enzyme GTP cyclohydrolase I (92). The synthesis of this enzyme is upregulated by IFN-γ. Further steps leading to production of tetrahydrobiopterin are catalyzed by enzymes that appear not to be under control of cytokines. The tetrahydrobiopterin concentration in cells is therefore mainly dependent on the level of GTP cyclohydrolase, although part of the primary product of this enzyme may also leave the cells after dephosphorylation. The latter escape pathway explains the increased levels of neopterin in urine and tissues of patients with inflammatory and infectious diseases.

4. *Generation of Nitrogen Monoxide*

Various cell synthesize and release endogenous NO as a short-distance and short-lived messenger (93). NO is produced by NO synthases, which convert L-arginine to L-citrulline and NO. NADPH is consumed in the reaction in stoichiometric proportion and tetrahydrobiopterin is required as a cofactor that does not seem to be involved stoichiometrically but is apparently required for each step of the reaction. The NO synthase reaction can be blocked, both *in vitro* and *in vivo*, by N^G-monomethyl-L-arginine (L-NMMA). The use of L-NMMA has helped a great deal in defining the role of NO in physiological and pathological processes. Production of NO is controlled by IFN-γ via two pathways: (a) regulation of the synthesis of the inducible isoform of NO synthase; and (b) control of the production of the cofactor, tetrahydrobiopterin [reviewed in ref. (92)].

NO synthase occurs in at least three isoforms. Two forms are "constitutive," being produced mainly by endothelial cells and platelets on the one hand and by neurons on the other hand. The constitutive NO synthase is tightly associated with calmodulin, which allows for regulation of the enzyme's activity by intracellular Ca^{2+} ion concentration. In particular, activity of the endothelial constitutive NO synthase is regulated by pulsatile blood flow and shear stress and can be enhanced by neurotransmitters, e.g., acetylcholine, bradykinine, and ATP. Certain neurons contain constitutive-type NO synthase [for review see Ref. (94)]. One location is the myenteric plexus of the gastrointestinal tract; these neurons govern peristaltic activity. Other locations are the nerve plexuses of brain and penile arteries. Regulated NO release by these neurons accounts for relaxation of cerebral arteries and for erection. NO is considered to be a neurotransmitter. However, in contrast to classical neurotransmitters, which are released by exocytosis of synaptosomes, NO is synthesized on demand and diffuses out of the cell.

a. NO Induction by IFN-γ. The inducible NO synthase occurs *in vitro* in mononuclear phagocytes, granulocytes, fibroblasts, Kupffer cells, hepatocytes, endothelial cells, vascular smooth muscle cells and, probably, in many other cell types. The enzyme is not detectable in uninduced cells and differs from the constitutive type in that its induced release requires protein synthesis, that it can act in the absence of Ca^{2+}, and that its activity is stimulated by flavin adenine dinucleotide and reduced glutathione.

In macrophages and fibroblasts, the known natural inducers of the enzyme are cytokines and endotoxin. Maximal activation of the cells to produce NO via this pathway is obtained by their exposure to a combination of IFN-γ and endotoxin, IFN-γ and TNF, or IFN-γ and IL-1. Macrophages of mice with a targeted disruption of the IFN-γ receptor gene were expectedly found not to produce NO in response to IFN-γ. Other cytokines, TNF and IFN-α/β, could only marginally substitute for IFN-γ, indicating that IFN-γ is indeed the major cytokine controlling the NO-generating pathways (95).

The promotor of the inducible NO synthase gene contains a response element for IRF-1, a transcription factor affecting IFN-γ- as well as IFN-α/β-responsive genes. Peritoneal macrophages of IRF-$1^{0/0}$ mice were found to produce little or no NO on stimulation with IFNs, and mRNA levels for the synthase remained unchanged. The IRF-1 defect in these mice was associated with a severely reduced resistance to infection with BCG (95). However, whether defective NO formation, rather than defects in other IRF-1-controlled events, explains this reduced antimycobacterial resistance remains to be defined.

b. Occurrence and Effects of NO in Biological Systems. NO exists in three equilibrating redox states (96)—nitroxyl anion (NO^-), neutral nitric

oxide ($NO^{\cdot}$, single electron in an anti-binding orbital), and the nitrosonium ion (NO^+)—that have different biological activities. $NO^{\cdot}$ reacts with (di)oxygen in its various redox forms (O_2, $O_2^{\cdot-}$, and $O_2^{2\cdot-}$) to generate $OONO^-$ (peroxynitrite) and $NO_2^{\cdot}$. $NO^{\cdot}$ also forms complexes with transition metal ions in metalloproteins, heme-containing ones such as hemoglobin, and proteins containing iron–sulfur centers such as those involved in mitochondrial electron traffic (aconitases and complex I and II). The formation of iron–dinitrosyl-dithiolate complexes in mitochondrial enzymes accompanies cytotoxicity-inducing effects of IFN-γ. NO^+ nitrosates organic molecules at S, N, O, or C centers. In biological systems, this may result in amine deamination ($R–NH_2 \rightarrow R–H$), *N*-nitrosylation ($RR'–NH \rightarrow RR'–N–NO$), or thionitrite formation ($RS^- \rightarrow R–S–NO$). In biological systems nitrosothiols predominate over other nitrosated organics. The existence of these different equilibrating pools of NO provides the means for regulation of transport, lifetime, and biological availability of NO.

The physiological receptor for NO is the soluble guanylyl cyclase, which generates cGMP. The enzyme is activated by binding of NO to its heme iron. Effects of NO mediated via this signaling pathway are vasodilation, platelet inhibition, cell adhesion, and neurotransmission. By nitrosylating free thiols, NO can regulate the activity of certain enzymes and thereby exert physiological regulatory functions. Thus, the reaction of NO with cell surface thiols has been associated with antimicrobial effects, modulation of ligand-gated receptor (NMDA) activity, and alterations of smooth muscle function. The antimicrobial effect of NO may also be due to loss of iron from infected host cells (97).

In vitro, cells producing NO, following exposure to IFN-γ, can die a suicide-like death (98, 99), in particular when they have no access to glucose or when glycolysis is blocked, so that the respiratory chain is the the only pathway for ATP generation. However, aside from being cytotoxic, NO can also exert cytostatic activity by causing arrest of DNA synthesis. This inhibition precedes inhibition of mitochondrial respiration and may be due to impairment of the enzyme ribonucleotide reductase, which contains at least three targets for NO: a tyrosyl radical, cysteines, and an iron center.

Macrophages in which NO production is induced by IFN-γ have cytocidal activity toward other cells in which no NO can be generated. Cell death in this case can be due to interruption of mitochondrial respiration (100), but generation of peroxynitrite may also be involved.

c. The Role of IFN-γ-Induced NO in Disease. Production of NO in phagocytic cells is associated with reduced survival of ingested microorganisms. The role of the IFN-γ-induced NO synthase is therefore assumed

to consist in augmented defense against infection with bacteria, molds, or protozoa [for review, see Ref. (101)]. NO released by activated macrophages is also held responsible for the ability of these cells to kill tumor cells and is therefore considered a defense mechanism against cancer. The usefulness of cytokine-induced NO release by other cells (fibroblasts, Kupffer cells, hepatocytes, etc.) is largely a matter of speculation. The cytotoxic effect of induced NO may also cause undesirable cell and tissue damage.

In view of the effects of NO on the vasculature, one may propose that NO produced by IFN-γ-activated macrophages is in part responsible for local vasodilation in the inflammatory focus and possibly for the hyperdynamic circulation response associated with inflammation. In animal models, beneficial effects of induced release of NO have been revealed by the administration of L-NMMA, which blocks synthesis of NO [for review see Ref. (93)]. Thus, administration of L-NMMA was found to potentiate endotoxin-induced intestinal damage as well as liver damage in a sepsis model and to abrogate the protective effect of a low endotoxin dose against subsequent challenge with a lethal dose (102). However, as a contrast, hypotension also occurring as part of the septic shock syndrome seems in part to be due to excessive production through the endotoxin/cytokine-induced pathway of NO.

There is circumstantial evidence that IFN-γ is involved in the pathogenesis of multiple sclerosis (see below). Therefore, it is of interest to note that NO release may be involved in demyelinative processes: microglial cells have the ability to kill oligodendrocytes in culture and the lysis was found to inhibited by antagonists of NO (103).

Similarly, NO induced by IFN-γ or other cytokines may be involved in the pathogenesis of insulin-dependent diabetes in NOD mice (see below). *In vitro,* IL-1β can kill β cells in certain circumstances. This cytocidal activity was found to depend on generation of NO as it could be prevented by L-NMMA (104).

Finally, NO production has also been found to contribute to the antiviral effects of IFN-γ in macrophages infected with ectromelia, vaccinia, or herpes simplex-1 viruses (105). In the macrophage-like cell line, RAW 264.7, IFN-γ-induced NO was found to inhibit vaccinia virus DNA replication, late viral protein synthesis, and particle assembly, but not to affect early protein synthesis. Virus replication was inhibited not only in the iNOS-producing macrophages themselves but also in bystander cells cocultured with the IFN-γ-pretreated macrophages (106). Remarkably, this apparently paracrine effect was not seen when the cells were separated from each other by a semipermeable membrane, suggesting that, in addi-

tion to NO, cell–cell contact is also necessary for transfer of the antiviral state.

5. *Other IFN-γ-Induced Proteins*

Aside from the IFN-γ-induced proteins and enzymes already mentioned, several others have been reported. Some of these proteins have been found serendipitously or by molecular screening strategies such as subtraction cloning. Often, the function of these proteins has remained unknown for at least some time. One example is the protein IP-10, originally identified by cloning a mRNA from cells treated with IFN-γ. At the time the sequence of IP-10 became known, no sequence-homologous proteins were known. Only when interleukin-8 and related molecules were isolated did it become evident that IP-10 belongs to the large family of chemokines (107).

Another example is the murine protein Mg21, identified by subtraction cloning of cDNA from peritoneal macrophages treated with IFN-γ (108). This protein appears to belong to a family of GTP-binding proteins that also encompasses IRG-47 (109), which is induced by IFN-γ in B lymphocytes, and Mx, which is an IFN-α/β-induced protein responsible for the antiviral effect against influenza virus. GBP-1 is a GTP-binding protein induced by IFN-γ in human fibroblasts (110) and in mouse macrophages (111); however, its sequence is unrelated to those of IRG-47 and Mg21.

V. Biological Effects on Cells and Tissues

A. The Antiproliferative Effect of IFN-γ

IFN-γ exerts a mild antiproliferative effect on most cell types (with the exception of at least some populations of activated T cells or T cell lines). One example of cells that are relatively sensitive to growth inhibition by IFN-γ is normal keratinocytes (112). As is the case with many other actions of IFN-γ, a synergy with TNF was found. IFN-γ is also a potent inducer of differentiation in keratinocytes. These effects are characterized by a dramatic reduction in expression of the mRNA of two growth-regulatory genes, cdc2 and E2F-1, and an increase in the expression of squamous cell-specific genes (113). The cytostatic effect of IFN-γ on keratinocytes may pertain to the epithelial atrophy seen in the skin of patients with GVH disease.

A dose-dependent and reversible antiproliferative effect of IFN-γ was seen to occur in a human submandibular salivary gland epithelial cell line (HSG). Concurrently, the expression of class II MHC gene product and ICAM-1 was increased. The effect may play a role in Sjögren's syndrome, which is characterized by an inflammatory cell infiltrate associated with

progressive atrophy of acinar secretory epithelium and loss of secretory function (114).

B. Expression of Membrane Proteins

One of the best documented actions of IFN-γ is the induction of MHC class II antigens on many but not all types in culture. IFN-γ thus has the ability to enhance or to induce these cells' ability to present foreign antigens. MHC class I antigen expression can also be enhanced under the influence of IFN-γ. Cells in which this effect occurs may thus become better targets for cytotoxic T cells recognizing viral, tumor, or autoantigens present in such cells.

In rats given IFN-γ injections, MHC class II antigen expression is seen to occur in all organs including the brain. This induced expression is rapidly lost after interferon withdrawal except in keratinocytes, in which it lasts for several days (115). Some cell types are completely resistant to induction of class II antigens, e.g., capillary endothelium, neurons, and endocrine islet cells. Human islet cells do, however, express class II antigens after treatment with IFN-γ + TNF (116), an effect that may play a role in the pathogenesis of autoimmune insulin-dependent diabetes.

IFN-γ regulates the expression on phagocytes of the high-affinity Fcγ receptor I (FcγRI). Resting monocytes express both FcγRI and the low-affinity receptors FcγRII and FcγRIII; resting polymorphonuclear cells express only the low-affinity receptors. IFN-γ induces expression of FcγRI on neutrophils (117) and augments its expression on mononuclear phagocytes (118). Ligand binding to FcγRs is widely recognized to stimulate effector functions of phagocytes, such as phagocytosis, tumor cell killing, and inflammatory mediator release. Therefore, augmented FcγR expression is one of the pathways by which IFN-γ can act as a proinflammatory cytokine. Isolated rat brain microglia also displays enhanced expression of Fc receptors after treatment with IFN-γ, TNF-α, or IL-1. Remarkably, the combination of IFN-γ and TNF-α inhibits Fc receptor expression (119).

Certain viruses, e.g., Dengue virus, enter host cells via specific antibodies bound to FcγR. As a consequence, IFN-γ paradoxically augments virus infection in mononuclear cells in the presence of antibody (120).

IFN-γ augments expression of FcεR on the human mononuclear cell line U937 (121) and on platelets (122). In view of the important role of IgE in resistance to parasitic diseases and in type I allergic reactions, these effects of IFN-γ need to be taken into account when considering the role of IFN-γ in these diseases (see below).

IFN-γ is among the cytokines that augment expression of the adhesion molecule ICAM-1 on various cell types, including cultured endothelial cells (see below) and epidermal keratinocytes (123), resulting in increased

adhesiveness for leukocytes expressing the integrin LFA-1. The significance of this effect may be that IFN-γ produced early in an aspecific inflammatory focus (e.g., by NK cells) is coresponsible for firm adhesion of granulocytes to endothelial cells in postcapillary veins as a prelude to their spreading and diapedesis. Similarly, this mechanism is believed to promote proximity of dendritic cells, epidermal keratinocytes, and lymphocytes during antigen presentation in the skin (123).

Another important membrane protein induced by IFN-γ is the B7 antigen, whose ligand on T cells is the CD28 molecule (124). The presence of B7 on antigen-presenting cells is indispensable for them to avoid delivering an anergizing signal (125).

IFN-γ has also been reported to augment expression and/or shedding of tumor-associated antigens by tumor cells, thereby modulating their targetability for the corresponding antibodies or sensitized T cells (126–128).

Bone marrow-derived macrophages express a protein with an epitope recognized by a monoclonal antibody specific for a peptide of the mycobacterial heat shock protein, hsp60. Exposure of the cells to IFN-γ was found to result in increased expression of this cross-reactive epitope. Antibodies recognizing heat shock proteins are believed to play a role in autoimmune diseases. Therefore, the augmenting effect of IFN-γ on expression of hsp60 cross-reactive proteins may be of relevance to the pathogenesis of such diseases (129).

C. Effects on Mononuclear Phagocytes

IFN-γ has long been recognized as the foremost important cytokine converting macrophages from a "resting" to an "activated" state. "Activation" is a rather indiscriminately used term that has meaning only if placed in a context of a well-defined function or functional ability that is considered. The mononuclear phagocytes (MPC) population, to which macrophages belong, comprises cells in different stages of differentiation and maturation, i.e., bone marrow precursors, blood monocytes, and different types of tissue macrophages (e.g., connective tissue histiocytes, alveolar macrophages, Kupffer cells, exsudate macrophages, microglial cells, osteoclasts, etc.). These stages are usually considered as steps in a process that, although regulated by environmental signals, is in essence irreversible. Activation states of tissue macrophages, as a contrast, are mostly seen as reversible changes determined by the temporally changing tissue microenvironment. IFN-γ and other cytokines, being constituents of the cellular microenvironment, play an important role both in the differentiation and maturation of MPCs and in activation of tissue macrophages.

Circulating monocytes, when placed in culture, undergo apoptosis unless provided with certain stimuli, e.g., LPS and/or certain cytokines. Typically, human blood monocytes will survive for more than 7 days in the presence of pure MCSF, whereas in its absence, they will die within 24 hr by apoptosis and secondary necrosis. In the presence of MCSF, the 100% surviving monocytes become progressively resistant against withdrawal of the growth factor and can subsequently be activated by exposure to a stimulus (e.g., serum-activated zymosan) to become biologically active (as evident from adherence, phagocytic, and respiratory activity). In the presence of not only MCSF but also IFN-γ, resistance to cytokine withdrawal develops more rapidly. Thus, although IFN-γ cannot by itself replace MCSF, it can be seen as a synergist for MCSF to avoid apoptosis and promote maturation into a macrophage. However, remarkably, such monocytes that have avoided apoptotic death in the presence of both MCSF and IFN-γ, as opposed to those that have maturated in the presence of only MCSF, do undergo apoptosis when subsequently exposed to an activating stimulus (130). The authors speculate that this yin-yang-type effect of IFN-γ fulfills a useful function in host defense against infection in that it allows for rapid development of a microbicidal macrophage, but also for subsequent elimination of the macrophage that, by producing toxic metabolites, might otherwise inflict undue damage on the hosts own tissues.

Macrophages activated by IFN-γ or other agents differ from resident ones by enhanced endocytic capacity, as manifested by increased pinocytosis and phagocytosis via receptors for complement and IgG2a. Such enhanced endocytic capacity does not, however, apply to all ligands. Expression of Fc receptors for other Ig classes, for instance, has been shown to be reduced in the activated state. Significantly, macrophages activated by IFN-γ have been found to have reduced ability to ingest a variety of obligately intracellular microorganisms, e.g., *Rickettsiae, Trypanosoma cruzi,* and *Leishmania* amastigotes. In the case of *Leishmania* in mouse macrophages, reduced binding was demonstrated to be due to reduced expression of lectin-like receptors for the organisms (131). Reduced uptake of intracellular parasites by IFN-γ-activated macrophages may represent one of the mechanisms by which IFN-γ contributes to both specific and aspecific host resistance to these pathogens.

IFN-γ also affects accessory cell function of mononuclear phagocytes. *In vivo,* endogenous IFN-γ seems to promote helper and to counteract suppressive circuitry, as evident from experiments in which anti-IFN-γ antibody was injected in carrier-primed mice whose spleen cells were subsequently immunized *in vitro* with carrier–haptene complex (132). However, IFN-γ may also activate suppression circuits in mixed mononuclear cell populations (133, 134).

IFN-γ regulates production of chemokines by macrophages. It is a potent inducer of IP-10 mRNA expression in human and murine macrophages (135, 136). IP-10 belongs to the chemokine-α family. Human IP-10 has been shown to exhibit chemoattractant activity for monocytes and activated T cells and to promote T cell adhesion to endothelial cells (107). On the other hand, IFN-γ was found to suppress LPS-induced expression of certain other related chemokines, namely MCP-1 and KC (GRO/melanoma growth-stimulating activity) in mouse peritoneal macrophages (137). Similarly, induction of IL-8 by IL-2 or IL-1 in human monocytes was found to be inhibited by IFN-γ (138). However, IFN-γ is not to be regarded as a general inhibitor of IL-8 expression, because it synergizes with TNF to induce IL-8 in other cells. Clearly, IFN-γ has the ability to either stimulate or inhibit production of proinflammatory mediators by macrophages.

IFN-γ induces production of C1 inhibitor protein (C1INH), both in mononuclear phagocytes *in vitro* and in the serum of patients given intravenous infusions *in vivo* (139, 140). C1INH is a 105-kDa glycoprotein considered to be the major inhibitor of several serine proteases including C1s, C1r, kallikrein, and activated Hageman factor. Hepatocytes, activated mononuclear phagocytes, and endothelial cells constitute the major source of C1INH.

D. IFN-γ and Antigen Presentation

IFN-γ can affect antigen presentation by augmenting expression of MHC class II molecules on membranes of professional antigen-presenting cells (APCs), but also by inducing *de novo* expression on the surfaces of various other cell types, thus converting them to nonprofessional APCs. The consequences of these effects are subject of debate. In particular, MHC class II-restricted antigen presentation by nonprofessional APCs that outnumber the professional ones is speculated to result in antigen-specific T cell anergy or induction of suppressor cells. Thus, IFN-γ was demonstrated to inhibit the ability of epidermal cells to present tumor-associated antigens for protective immunity, as well as the ability of the epidermal cells to elicit tumor-specific DTH in tumor-immune mice *in vivo* (141). One possible underlying mechanism for anergy to be induced by nonprofessional APCs may be that these cells lack critical costimulatory cell surface molecules such as ICAM-1 (142), B7 (125), or their ligands. These molecules do occur on professional APCs, and their expression is enhanced by IFN-γ (124).

T cell anergy has also been proposed to occur when antigen fragments occurring in high concentration in tissues are captured and presented in MHC class II context by T cells (143). However, there is no evidence that IFN-γ would induce expression of MHC class II molecules on T cells.

E. Effects on Endothelial Cells

Evidence from a large number of studies indicates that IFN-γ induces expression of MHC class II antigen on endothelial cells *in vitro* and *in vivo*. Increased class II antigen expression following IFN-γ treatment is also seen in monolayers of cerebral vascular endothelia, which are endowed with unique barrier properties, as they differ from extracerebral endothelia by the presence of interendothelial tight junctions, by a paucity of cytoplasmic vesicles, and the presence of specialized membrane transport systems. Exposure of monolayers of such cells to IFN-γ is followed by morphological alterations associated with increased permeability of confluent monolayers to macromolecules (144).

IFN-γ, as well as IL-1 and TNF-α, augments expression of the adhesion molecule ICAM-1 on cultured endothelial cells of extracerebral (145) as well as cerebral origin (146) resulting in increased adhesiveness for leukocytes expressing the integrin LFA-1 (147). In the rat, an antigen (4A2) has been identified that is induced on cerebral endothelial cells after exposure to IFN-γ. Triggering of the antigen with its corresponding monoclonal antibody was found to augment lymphocyte adhesion via LFA-1 and possibly other integrins (148).

IFN-γ enhances the capacity of endothelial cells for producing IL-1 in response to LPS (149–151). A similar effect has been noted in cultures of synovial cells (152).

Chemokine secretion by endothelial cells is under regulatory control by cytokines, in particular IFN-γ and TNF-α: barely any RANTES was found to be produced by human vascular endothelial cell cultures treated with IFN-γ, TNF-α, or IL-1β. However, the combination, TNFα + IFN-γ, was highly effective in inducing RANTES production: pretreatment with IFN-γ sensitized the cells to induction with TNF-α. This regulatory control was found to be exerted at the transcriptional level (153).

F. Synergy between IFN-γ and TNF

Many instances have been reported in which IFN-γ synergizes with TNF either *in vitro* or *in vivo*: *in vitro* cytotoxicity for certain tumor cells, induction of microbicidal activity in macrophages, induction of NO release by various cell types, expression of cell surface adhesion molecules, *in vivo* antitumor effects, *in vivo* induction of other cytokines, systemic toxicity, and lethality (for references, see relevant sections).

The subcellular mechanism(s) underlying this synergy are poorly understood. IFN-γ has been found to augment expression of TNF receptors by certain cell lines (154–156). However, this is not the general rule and cannot explain all synergistic effects described. In explanted murine perito-

neal macrophages, IFN-γ, when added at relatively high concentrations, was found to inhibit rather than enhance expression of TNF receptors: in freshly explanted macrophages it prevented the appearance of receptors, and in mature macrophages it downregulated receptors already expressed (157). This inhibitory effect was not considered to be contradictory to the synergy between TNF and IFN-γ because such synergy was only seen at lower doses of IFN-γ than those inhibiting TNF receptor expression. This dose-dependent difference in interaction between the two cytokines may be of particular importance for the interpretation of often contradictory effects of both cytokines seen in inflammatory models *in vivo*. It should also be mentioned that in these studies no distinction was made between effects of IFN-γ on the low- versus the high-molecular-weight receptor of TNF.

G. Natural IFN-γ Antagonists

Several cytokines interact with the IFN-γ system in a synergistic fashion either by enhancing its production or its action (Fig. 3). The biological significance of these synergisms is commented on in several sections of this review. On the other hand, several cytokines act antagonistically toward IFN-γ and can thereby counterbalance its disadvantageous actions (Fig. 4).

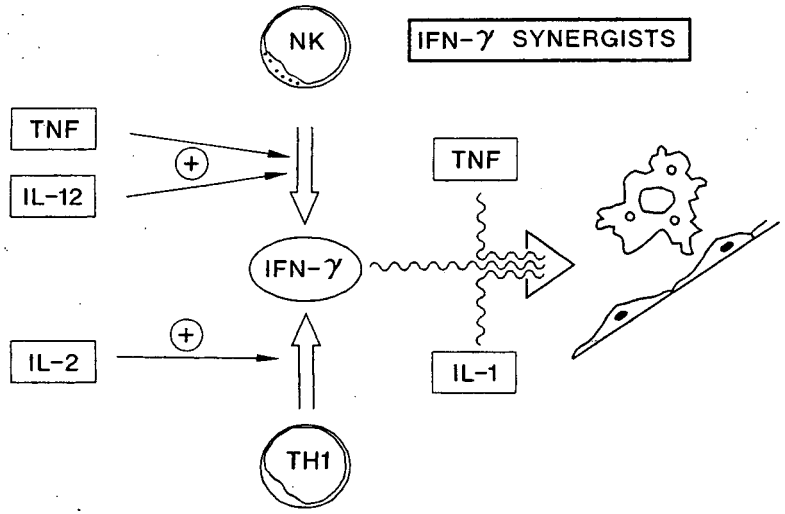

FIG. 3. Synergists of IFN-γ. Several cytokines can reinforce the production or the action (or both) of IFN-γ. IL-12 and IL-2 stimulate IFN-γ production by, respectively, NK cells and T cells. The monokines, TNF and IL-1, reinforce the actions of IFN-γ, in particular those exerted on mononuclear phagocytes and endothelial cells, but also those exerted on thymocytes and lymphocytes.

1. *Interleukin-4*

IL-4 [for review see Ref. (158)] is produced by stimulated T helper cells, in particular the TH2 subset. It affects mainly B and T lymphocytes. Thus, IL-4 stimulates growth and membrane receptor expression on B lymphocytes elicited by other agents. It also acts as an isotype switch factor in favor of IgE and IgG1 in the mouse and of IgE in man. IL-4 may also play a role in the development of B cells by promoting differentiation of stem cells into B lymphocyte rather than myeloid lineage. IL-4, in conjunction with other cytokines, favors the growth of TH2 cells, the subset which is also the source of IL-4. It appears to have no effect on proliferation of TH1 cells but inhibits their IFN-γ production. IL-4 does promote growth and differentiation of cytotoxic T cells and plays an important role as a growth inhibitor for immature thymocytes and a maturation promoting factor for $CD4^+/CD8^+$ cells in the thymus (159).

In several systems, IL-4 and IFN-γ have been found to have opposite effects and to antagonize each other when they are both present. Typical effects of IFN-γ on monocytes and macrophages (induction of IFN-γ-

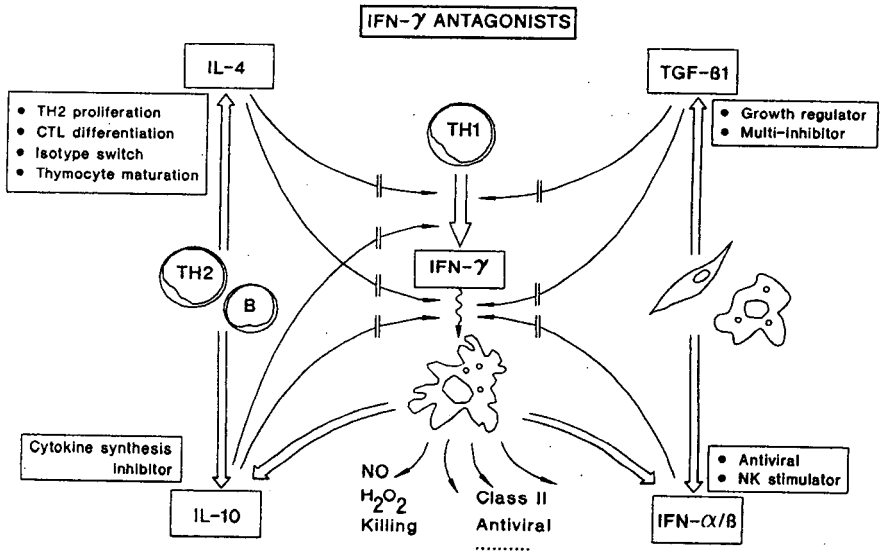

FIG. 4. Antagonists of IFN-γ. Both the production and the actions of IFN-γ are kept in check by several other cytokines, which have their origin in lymphocytes (IL-4, IL-10) as well as in fibroblasts and mononuclear phagocytes (IFN-α/β and TGF-β1).

responsive genes, H_2O_2 production, and intracellular antimicrobial activity) are counteracted by IL-4 (160, 161), although synergy has also been reported (162). Also, the antiviral affect of IFN-γ on L929 cells has been found to be antagonized by IL-4 (163). IL-4 inhibits induction of chemokines by IFN-γ (153, 164).

In reverse, typical IL-4 actions on B lymphocytes (isotype switch in favor of IgE) and T lymphocytes (inhibition of thymocyte proliferation) are counteracted by IFN-γ (159, 165, 166). Finally, IL-4 antagonizes with IFN-γ by inhibiting its production by peripheral blood mononuclear cells (167–169).

The subcellular mechanism of antagonism between IFN-γ and IL-4 in monocytic cells involves inhibition by IL-4 of transcriptional activation of the IFN-γ-inducible proteins as was shown to be the case for the chemokine IP-10. The target for inhibition seems to be the ISRE in the promoter of the IP-10 gene (164). However, rather than inhibiting activation of the ISRE-binding transcription factor, ISFGF-3, IL-4 seems to induce a distinct ISRE-binding factor that functions to inhibit IFN-γ-driven, ISRE-dependent transcription (170).

The production of IFN-γ by antigen-stimulated $CD4^+$ T cells depends on the presence of IL-2 during both the priming and the expression phases. In contrast, production of IL-4 requires IL-2 only during the priming phase. This difference in requirement of IL-4 and IFN-γ production may be part of the mechanism directing the immune response toward TH1 or TH2 predominance.

2. *IFN-α/β and TGF-β1*

Type I interferon (α or β) counteracts induction by IFN-γ of MHC class II antigens in murine macrophages (171, 172). The antagonistic effect has also been observed in cultured human astrocytes (173) and astrocytoma cells, but not in human monocytes (174). As documented by nuclear run-on experiments, the antagonistic effect is exerted at the transcriptional level (174).

TGF-β1 is a 25-kDa, disulfide-linked protein that has either growth-enhancing or growth-inhibitory properties depending on the cell type and the presence of other growth factors. TGF-β1 is secreted, as a latent protein complex, by a variety of cells including lymphocytes, platelets, and activated macrophages. Once activated, it binds to a complex set of receptor proteins present on many different cell types. On immune cells, TGF-β exerts a variety of mostly inhibitory effects: it inhibits IL-2-dependent T cell proliferation, expression of IL-2R, B cell proliferation and differentiation, IL-1-induced thymocyte proliferation, and cytotoxic lymphocyte generation. Although TGF-β1 can by itself induce transcription of cytokine

genes, it mostly inhibits production of cytokines, such as IL-1β, TNF, and IFN-γ, induced by other agents such as LPS or PHA (175, 176). The mechanism of this inhibitory effect remains unclear; synthesis of PGE2 or cAMP, which have both been implicated in post-transcriptional control of cytokine expression, do not seem to be involved.

TGF-β1 can also counteract IFN-γ action as it was found to downregulate constitutive as well as IFN-γ-induced expression of MHC class II antigens on a human melanoma cell line (177). Another IFN-γ-controlled activity that is counteracted by TGF-β is induction of nitrogen oxide synthesis in macrophages (178). In reverse, IFN-γ can antagonize actions of TGF-β. Thus, it has been reported that IFN-γ reverses the stimulation by TGF-β of the collagen gene but not that of the fibronectin gene expression in normal human fibroblasts (179).

3. *IL-10*

Interleukin-10 [for review, see Ref. (180)] was originally described as "cytokine synthesis inhibitory factor" produced by TH2 cells. However, monocytes and B lymphocytes can also produce IL-10. Production by mononuclear phagocytes seems to be induced mainly in an autocrine fashion by TNF-α, and may represent an important negative feedback pathway (181). Indeed, although IL-10, like all cytokines, possesses multiple biological activities, its main effect remains its capacity to inhibit the synthesis of IFN-γ. In addition, however, IL-10 also counteracts the action of IFN-γ, e.g., as it inhibits IFN-γ-induced nitrogen oxide production (182). The powerful inhibitory effect of IL-10 on IFN-γ production is illustrated by the observation that IL-10 administration to mice can inhibit shock induced by SEB in mice (183).

The antagonistic effect of IL-10 on IFN-γ is reciprocal. IL-10 production by monocytes is inhibited by IFN-γ (184). It seems likely, therefore, that the balance between IL-10 and IFN-γ in the initial stages of an immune response is of crucial importance to determine the further course of the response. For instance, selective stimulation of IL-10 production has been proposed as a strategy used by certain microorganisms, in particular helminths and protozoans, to evade the IFN-γ-mediated host defense.

4. *Interleukin-13*

IL-13 is a TH2 cell-produced cytokine whose amino acid sequence is partly homologous to that of IL-4 [for review see Ref. (185)]. IL-13 and IL-4 also share some biological properties, one of them being a modulatory effect on activated macrophages. Treatment of activated macrophages with IL-13 reduces production of inflammatory monokines in response to

IFN-γ or LPS. In addition, IL-13 also inhibits production of nitrogen monoxide by activated macrophages (186).

5. *Tumor Necrosis Factor and Interleukin-6*

TNF-α synergizes with IFN-γ in many *in vitro* test systems. However, this is by no means a general rule. With regard to two important activities, enhancement of expression of MHC class II molecules and of Fc receptors, the combination of IFN-γ and TNF-α has been reported to be less active than either cytokine alone, both in rat peritoneal macrophages and in microglia (119, 187). In fact, whether IFN-γ and TNF-α synergize or antagonize with each other may critically depend on the state of differentiation of the cells. Thus, TNF was found to enhance IFN-γ-induced MHC class II molecule expression in undifferentiated macrophages and to inhibit such enhancement in mature macrophages (188).

IL-6 has been found to act as an antagonist for the *Toxoplasma*cidal activity of IFN-γ in murine peritoneal macrophages *in vitro* (189). Combining TNF-α with IL-6 pretreatment resulted in restoration of the *Toxoplasma*cidal activity, whereas addition of anti-TNF-α antibody to this combination resulted in enhancement of the IL-6-mediated impairmant of IFN-γ function. It would appear, therefore, that in this system IFN-γ and IL-6 interact at the level of TNF-α triggering.

H. IFN-γ and Lymphocytes

1. *Effects on T Cell Proliferation and Apoptosis*

Whereas IFN-γ acts as a mild inhibitor of proliferation for most cell types, it stimulates proliferation of mitogen-triggered primary T cells as well as a variety of T cell lines [see Ref. (190)]. Remarkably, the antiviral effect of IFN-γ is not expressed in these cells. It has been considered that this exceptional situation is due to modulation of signal transduction in T cells and that enhancement of IL-2 production and IL-2R expression are involved (190). In studies using murine T cell clones, it was found that IFN-γ exerts a slight suppressive effect on IL-2- and IL-4-mediated proliferation of TH2 but not TH1 clones (191, 192). In mice, IFN-γ was shown to mediate the inhibitory effect of IL-12 on TH2 cell-mediated pathology (193). In fact, a basic difference between TH1 and TH2 cells appears to be the absence of the β-chain of the IFN-γ receptor in the TH1 population (194).

One aspect of the regulatory effect of IFN-γ on lymphocytes is its ability to promote apoptosis under specific conditions. Thus, blockage of IFN-γ inhibits cell death induced in effector T cells by TCR linkage in the absence of accessory cells (195). Furthermore, both in normal and in cultured malignant lymphocytes, IFN-γ has been shown to exert contrasting effects, i.e., apoptosis or proliferation, depending on the level of expression of IFN-γ receptors: high-level expression is associated with an apoptotic response, low-level expression with a proliferative one (196). *In vivo* apoptosis of thymocytes after treatment with anti-CD3 antibody is more pronounced in mice that are deficient in expression of the IFN-γRα chain than in their wild-type counterparts, indicating that in this system IFN-γ triggers an antiapoptotic pathway (197).

2. *IFN-γ and the Generation of Cytotoxic Lymphocytes (CTLs)*

Exogenous IFN-γ can augment the development of CTL activity in mixed lymphocyte cultures, and neutralizing antibodies to IFN-γ can inhibit the development of CTL activity in antigen- or lectin-stimulated lymphocyte cultures [e.g., Ref (198)]. Anti-IFN-γ was also found to inhibit both proliferation and activation of CTL in the primary *in vitro* mixed-lymphocyte reaction (199, 200), an observation not subsequently confirmed under apparently similar conditions (201). A contribution of IFN-γ to the generation of CTL may, in principle, result from stimulatory effects on mononuclear phagocytes, on helper T cells, or on CTL precursors, or from inhibitory effects on suppressor T cells. In human mixed-lymphocyte cultures, the augmentation by IFN-γ of CTL generation was found to result mainly from a direct effect on $CD8^+$ T cells (198). In a system suitable for the expansion of single murine $CD4^-/CD8^+$ T cells into clones, both IL-2 and IFN-γ were found to be required (202).

3. *Effects of IFN-γ on B Cells*

Reports on the effects of IFN-γ on B cells are somewhat contradictory. In early studies, recombinant murine IFN-γ was found to possess B cell maturation factor activity for resting splenic B cells and the comparable B cell tumor line WEHI-279.1 (203). However, IFN-γ was found to inhibit LPS-induced IgM production in murine spleen-derived B cells by reducing the number of IgM-forming cells and without affecting overall proliferation (204). In fact, in B cells, as opposed to T cells, the stage of differentiation seems to codetermine the type of response to IFN-γ. Resting B cells seem to be unaffected by IFN-γ, whereas preactivated B cells are inhibited from further differentiation. These cells also show increased expression of IFN-γ receptors (204, 205). Also, cultured normal murine pre-B cells (206, 207) or human pre-B cell lines (208) that are exposed to IFN-γ undergo apoptosis, whereas human $CD5^+$ chronic B lymphocytic leukemia cells are protected from apoptosis by IFN-γ (209).

I. IFN-γ and Antibody Formation

IFN-γ affects antigen-presenting cells, T cells, and B cells, each of which intervene in the complex chain of events that leads from antigen exposure

to antibody production. No wonder that experiments testing the effect of IFN-γ on antibody formation have yielded disparate results depending on the experimental conditions—stimulus (antigen or mitogen), immunization system (*in vitro* or *in vivo*), immunization schedule, and isotype considered.

IFN-γ augments expression of MHC class II antigen expression and may therefore be expected to facilitate antibody induction in systems in which antigen presentation is the limiting factor. In murine *in vitro* immunization systems testing for primary IgM antibody responses to sheep erythrocytes, blockage of endogenous IFN-γ with antibodies that neutralize macrophage activation by IFN-γ have indeed been found to reduce the amount of antibody produced (210). IFN-γ was also found to be a necessary component of T cell-derived helper factors for antibody induction in *in vitro* immunization systems (211).

In contrast to this immunization-promoting effect of endogenous IFN-γ of which only minute amounts are actually detectable during the process, exogenous IFN-γ suppresses early antibody formation (210).

In cultured human PBMC, addition of IFN-γ has been found to promote and anti-IFN-γ antibody to inhibit spontaneous late production of IgG2; in PWM-stimulated cultures, exogenous IFN-γ inhibited and anti-IFN-γ stimulated early production of IgG1 (212). In this system, IFN-γ did not seem to act as a IgG2 switch factor because the IgG2-promoting effect disappeared when the culture system had been depleted of $sIgG2^+$ cells. However, in a culture system in which T cells are eliminated, IFN-γ seemed to possess IgG2-switching activity (213).

IFN-γ has also been found to stimulate polyclonal Ig production by resting or activated human B cells (203, 214).

IFN-γ and IL-4 antagonize each other in a variety of systems, and it has become general knowledge that antibody responses depend on the balance between two categories of cytokines, IFN-γ and IL-2 belonging to the first one (the TH1 cassette) and IL-4, IL-5, IL-6, and IL-10 belonging to the second one (the TH2 cassette). In this setting, the role of IFN-γ consists in suppressing IgG1 and IgE antibody formation and stimulating IgG2a antibody formation (215, 216). Following infection with influenza virus (217), the virus-specific IgG1 response was found to be significantly higher in IFN-γ-deficient than in normal mice, probably reflecting increased production of IL-4. Treatment with neutralizing anti-IFN-γ antibody in mice vaccinated with influenza virus antigens resulted in increased levels of antigen-specific IgG1 and IgE but reduced levels of IgG2 and IgG3 (218). In IFN-γ receptor knockout mice, the IgG1 antibody response to ovalbumin was not different from that in normal mice, but the IgG2a isotype was reduced (219).

When antigen in adjuvant (oil or alum) is injected, a very early change (Day 3) is the appearance at the injection site of IFN-γ-producing NK cells. Only later (Day 7) do cytokine-producing T cells appear in draining lymph nodes. At this site, IL-2 and IL-4 predominate over IFN-γ (220). The possibility is considered that early IFN-γ production by NK cell is in fact secondary to IL-12 production by macrophages that respond to the antigen and/or the adjuvant. Early IL-12 and IFN-γ may therefore play the crucial role in directing the immune response toward TH1 or TH2 predominance.

Association of increased IgE levels with imbalance between IL-4 and IFN-γ has been suggested to be involved in the pathogenesis of elevated IgE levels observed in patients with hyper-IgE recurrent infection (HIE) syndrome (221), atopic dermatitis, or helminth infections. In HIE, increased IL-4 production has not been observed. Conversely, some but not all studies reported decreased production of IFN-γ. Mitogen-driven IL-4 production by PBMNC of atopic subjects was found to be higher and production of IFN-γ lower than that of PBMNC of normal subjects (222). In patients with helminth infections, IgE levels were found to correlate with increased IL-4 and decreased IFN-γ production by parasite antigen-stimulated lymphocytes (223). However, in a placebo-controlled trial, exogenous IFN-γ failed to affect clinical parameters or IgE levels in patients with hay fever-type rhinitis due to ragweed allergy (224). In contrast, in a murine model for allergen sensitization, nebulized but not parenteral IFN-γ was found to decrease IgE production and to normalize airway function (225).

Elevated levels of IgE are among the immunological characteristics of chronic atopic eczema. IFN-γ responses have been reported to be defective in these patients (226). However, other changes are diminished delayed hypersensitivity reactions, diminished *in vitro* responsiveness to mitogens and recall antigens, and a decreased proportion of $CD8^+$ T cells. In a double-blind, placebo-controlled trial (227), replacement therapy with recombinant IFN-γ has been found to result in improvement of the clinical parameters. However, therapeutic failures in children with severe refractory disease have also been reported (228).

J. IFN-γ as a Mediator of Immune Suppression

In a variety of experimental systems, IFN-γ has been identified as a mediator of suppression of immune responses. GVH, a major complication of bone marrow transplantation, is associated with suppression of cellular immune responses, as evident from reduced proliferative responses of lymphocytes to mitogens. Addition of monoclonal antibodies against IFN-γ has been shown to relieve suppression, implying that endogenous

IFN-γ is involved (229). The target cell for IFN-γ in this system is believed to be a so-called natural suppressor cell (230). Other suppressor circuits induced by IFN-γ are those evident from inhibition of antibody production and general immunosuppression associated with parasite infections (see relevant sections).

Suppression by IFN-γ, as demonstrated by inhibition of proliferative responses of T cells, depends on the presence of mononuclear phagocytes. In fresh adherent mouse spleen macrophages as well as in macrophage hybridomas, the ability to suppress is MHC class II antigen-restricted and specifically depends on expression of I-J region-coded antigens, which are stimulated by IFN-γ (133, 134). A possible mechanism of suppression by mononuclear phagocytes is generation of H_2O_2 and prostaglandins because both catalase and indomethacin can alleviate suppression (231). Boraschi *et al.* (232), on the other hand, found that IFN-γ reduces rather than stimulates murine macrophage suppressive activity by inhibiting prostaglandin E2 release and inducing IL-1 induction. Another pathway used by suppressor macrophages, which can be activated by IFN-γ, is the generation of nitric oxide (233, 234). IFN-γ also enhances release by mononuclear phagocytes of TGF-β (235), which is generally known as an antiinflammatory cytokine.

K. IFN-γ and Epithelial Barriers

Epithelia represent an example of tight but flexible permeability barriers within the body. In an *in vitro* model, IFN-γ was shown to modulate permeability of an epithelial layer. IFN-γ was found to be rather unique in this respect, as the effect was not seen with IFN-α. Also, only the basolateral side and not the apical side of the epithelial layer was found to be responsive (236).

Most epithelial cells contain cytokeratins, a family of proteins forming cytoskeletal filaments. The expression of at least one of these molecules, the acid cytokeratin K17, was shown to be enhanced in HeLa cells treated with IFN-γ (237), and the promoter of this protein was found to contain three putative GAS elements (238). However, the exact mode of action of the promoter remains to be elucidated.

L. IFN-γ and Connective Tissue

Maintenance of intact and remodeling of traumatized or inflamed connective tissue is increasingly recognized to be controlled in part by the cytokine network. The contribution of IFN-γ to this control mechanism derives at least in part from its ability to inhibit the synthesis of collagen and fibronectin by fibroblasts or chondrocytes *in vitro* (239, 240). This inhibition is associated with reduction in mRNA levels (241, 242). Systemic

administration of IFN-γ was found to inhibit collagen synthesis in murine models—tissue reaction to a subcutaneous foreign body (243) or to skin wounds (244) and to alleviate pulmonary fibrosis induced by bleomycin (245).

M. IFN-γ and Adipocytes

Dissipation of fat stores during cachexia may in part be due to direct effects of IFN-γ (and other cytokines) on adipocytes. For instance, treatment of cultured adipocytes to IFN-γ results in a reduction of the amount of lipoprotein lipase activity releasable from the cells by incubation with heparin (246, 247). This enzyme is responsible for hydrolysing the triglycerides in circulating lipoproteins and is a major determinant of fat accumulation in adipocytes. IFN-γ has also been shown to reduce the rate of fatty acid synthesis (246, 247) and to cause increased hydrolysis of endogenous intracellular triglyceride in adipocytes (247, 248). In accord with these effects, IFN-γ was found to reduce the level of lipoprotein lipase and fatty acid synthetase mRNAs. However, the level of Ac–CoA carboxylase mRNA was found to remain unaffected and the level of hormone-dependent lipase mRNA was decreased rather than increased. Therefore, post-transcriptional as well as transcriptional regulatory pathways seem to be involved (247).

N. IFN-γ and Central Nervous System (CNS) Cells

IFN-γ influences the expression of membrane molecules in CNS cells, as it does in many other cell types. Class I and II MHC antigens are barely detectable in the normal human CNS. By contrast, in active lesions of multiple sclerosis and certain other neurological diseases (e.g., AIDS dementia complex or Alzheimer's disease), these antigens are prominently expressed on certain brain cells, especially microglia. Another membrane molecule induced by IFN-γ is ICAM-1. In MS lesions, small CNS vessels, mononuclear cells, and some glial elements contain ICAM-1 (249). Active CNS lesions in MS patients also contain cells producing several cytokines including IFN-γ (250).

The two main types of CNS targets for IFN-γ are microglia and astroglia. Microglial cells fulfill such functions as antigen presentation, phagocytosis, cytocidal activity, and production of tissue-degradative activity. Astrocytes act as regulators of the ionic balance in the CNS and neurotransmitter distribution. When cultured *in vitro,* these cells spontaneously express MHC antigens. Exposure to IFN-γ greatly enhances this expression. However, in astrocytes, as opposed to microglia, the effect of IFN-γ can be counteracted by various factors including IFN-β, IL-1, TGF-β, glutamate, and cAMP agonists, as well as contact with neurons. Normal human CNS

astrocytes, when put in culture, spontaneously express MHC antigens as well as ICAM-1. However, this expression is greatly increased in the presence of IFN-γ (251–256). The enhancing effect of IFN-γ on expression of class II MHC antigen, but not that on expression of class I MHC antigen or ICAM-1, was found to be counteracted by IFN-β (173, 257).

The molecular mechanism of class II MHC antigen induction in rat astrocytes and microglia has been studied in some detail. The class II MHC gene promoter DRA contains four conserved *cis*-acting elements—W, X_1, X_2, and Y—necessary for constitutive and induced transcription. Each of these elements binds to DNA-binding factors that are present in various cells. Also, a non-DNA-binding protein, CIITA, is necessary for expression of class II MHC genes. X_2 binds to a factor, IFNEX, which is induced by IFN-γ in astrocytes (258). A related factor was found to be operational in class II MHC gene induction in microglia (259).

In a murine oligodendroglioma cell line (MOCH-1), IFN-γ was found to induce a morphological change from a small round cell with thin branches to a large fibroblast-like cell. IFN-γ also stimulated markedly enhanced expression of the astrocyte marker protein GFAP (260).

O. IFN-γ in Hematopoiesis

Effects of IFN-γ on hematopoiesis have been demonstrated in many studies using different experimental settings, which invariably employ one or several hemopoietic factors and or cytokines. The question as to whether the IFN-γ effects depend on the presence of accessory cells (stroma cells, monocytes, or lymphocytes) has been a matter of controversy in early studies (261, 262) and can as yet not be considered resolved. Also, IFN-γ induces or enhances production of various other cytokines, and its effects on hematopoiesis may therefore be indirect. For instance, the IFN-γ-inducible chemokine IP-10 has been shown to inhibit colony formation from early hematopoietic progenitors, apparently by counteracting r-steel factor (rSLF) (263).

1. Effect on Hematopoietic Progenitors and Myelopoiesis

In bone marrow cultures prepared from normal mice, IFN-γ was found to inhibit granulocyte/macrophage colony growth. However, in cultures from mice pretreated with 5-fluorouracil it promoted colony formation. It was suggested that primitive progenitors require stem cell factor in the early stages and IFN-γ for subsequent growth (264). In the human system, IFN-γ has similarly been shown by many studies to counteract the proliferative activity of colony-stimulating factors. However, in cultures of pure $CD34^+$ progenitor cells, IFN-γ has been found to synergize with IL-3 (265). IFN-γ by itself did not affect proliferation, and in the presence of IL-3, although augmenting the number of colonies, failed to affect their size, indicating that it acted only on early progenitors. In fact, in long-term cultures, once mature cells appeared, IFN-γ inhibited further proliferation.

2. Erythropoiesis

IFN-γ has been found to suppress erythropoiesis both *in vitro* and *in vivo*, an effect that may play a role in anemia that accompanies chronic infections or autoimmune diseases. In affecting erythropoiesis *in vivo*, IFN-γ undoubtedly interacts with several other hemopoietic factors and cytokines. Studies have been aimed at revealing the most important of these interactions and at defining the most sensitive stages of erythropoiesis. In Epo-containing human CFU-E or BFU-E cultures, addition of IFN-γ was found to inhibit colony formation, an effect that could be reversed by increasing Epo concentrations (266). Similarly, in murine macrophage-containing bone marrow cultures supplemented with Epo, addition of IFN-γ was found to suppress formation of both BFUE and CFUE colonies (267). However, the dose required was lower for suppression of BFUE, and the overall effect was more pronounced the earlier IFN-γ was added after culture initiation. The effect of IFN-γ was not prevented by addition of single or combined antibodies against TNF-α, IL-1α, or GM-CSF. Accordingly, increased production of these cytokines in IFN-γ-treated cultures or synergy of these cytokines with IFN-γ did not seem to play a significant role in these macrophage-containing cultures. Nevertheless, in macrophage-depleted cultures, IFN-γ was shown to synergize with the antierythropoietic effect of TNF-α. Therefore, and also because cytokines other than those that were examined may be involved, it should not be concluded that IFN-γ exerts a direct inhibitory effect on BFU-E colony formation independently of other cells and cytokines.

P. Effects on Endocrine Cells

1. Thyroid

In cultured human thyrocytes, IFN-γ has been found to inhibit expression of thyrotropin receptors (268), production of triiodothyronin (269), and transcription of the thyroglobulin gene (270). It also reduces basal thyroid peroxidase content and inhibits thyrotropin-induced increase of the enzyme (271). Similar effects have been noted to occur in a rat thyroid cell line (FRTL-5), although the effects were minor unless IFN-γ was given in combination with TNF (272). These effects may in part explain the decrease in thyroid function in autoimmune thyroiditis.

2. Hypophysis

The effects of IFN-γ on the function of the anterior pituitary have been studied in an *in vitro* system consisting of organotypic anterior pituitary

cell aggregates (273, 274). In such aggregates, secretion of prolactin and ACTH in response to hypothalamic stimulatory factor was found to be inhibited by IFN-γ. This inhibitory effect was, however, codetermined by the composition of the aggregates. In particular, it required the presence of the folliculostellate (FS) cell component. Secretion of anterior pituitary hormones is known to be regulated by a variety of signals received from distant sites through the blood stream (endocrine) and from the hypothalamus (neuroendocrine). In addition, these signals are integrated into a local anterior pituitary network consisting of paracrine factors and perhaps cell-to-cell contacts (275). The cell populations in the anterior pituitary comprise diverse secretory elements, each producing its own set of hormones, but also comprise nonsecretory cells, in particular the FS cells. The stellate appearance of these cells results from their multiple cytoplasmic protrusions that embrace surrounding secretory elements grouping them into follicle-like aggregates. In *in vitro* systems, the FS cells have been shown to mitigate responses of secretory cells to stimulatory as well as inhibitory hypothalamic signals. One of their *in vivo* functions may therefore consist of avoiding overly brisk fluctuations in hormone levels. The FS cells also have properties and activities that resemble those of mononuclear phagocytes or glial cells—expression of typical macrophage and dendritic cell markers, phagocytosis, and secretion of IL-6 (276).

The apparent kinship of FS cells with other typical IFN-γ target cells and the FS cell-dependent inhibitory effect of IFN-γ on the anterior pituitary secretory activity invites speculation that IFN-γ, produced during inflammatory or immune responses, uses the FS cell to act on the neuroendocrine axis (277).

VI. Role of IFN-γ in Infection and Cancer

The role of IFN-γ in infection and cancer has been a subject of many studies that will be reviewed separately. Here, only the most salient facts and points of current discussion are mentioned.

IFN-γ is generally assumed to play a primordial role in defense against intracellular bacteria and parasites. In fact, many of these pathogens have their habitat in mononuclear phagocytes. They use the intracellular environment as a shield against microbicidal antibodies and specific host defense against them is therefore mainly dependent on cellular immunity mechanisms. Early during infection, IFN-γ is produced by NK-like cells; later on, by antigen-specific T cells, in particular $CD4^+$ cells that recognize microbial antigen on the class II antigen-positive infected phagocytes. This IFN-γ then triggers several microbicidal mechanisms in infected phagocytes as well as other cells, e.g., tryptophane oxidase and reactive

oxygen and nitrogen (see relevant section). It should be noted, however, that this cytokine-dependent pathway is complemented by a cytotoxic T cell pathway, which kills phagocytes or other cells that harbor microbial pathogens (278). Another type of mechanism by which early IFN-γ production is conceived to determine outcome of infections is by regulating the balance between TH1- and TH2-type responses (279, 280).

Remarkably, there are also distinct exceptions to the general rule that endogenous IFN-γ exerts a beneficial effect. Certain agents seem to have adapted so well to the immune system of the host that they have succeeded in perverting the cytokine network so that IFN-γ acts to their advantage. Such is the case in *Trypanosoma* infections in which endogenous IFN-γ accounts for generalized immunosuppression that accompanies the disease (281). Another mechanism by which endogenous IFN-γ acts to the disadvantage of the infected host is by being produced in excessive quantities. Such IFN-γ may enhance the production of other cytokines, e.g., TNF-α, which may cause tissue damage and death, or may act synergistically with such cytokines. One example is shock caused in mice by endotoxin, which is aggravated by endogenous as well as exogenous IFN-γ (282–284).

IFN-γ has the potential to protect the host against virus infection by virtue of its direct antiviral effect on most types of cells and by its regulatory activity on immunocytes. Not unexpectedly, exogenously administered IFN-γ has been found to act prophylactically against a variety of experimental virus infections, e.g., murine CMV infection in mice (285) or rat CMV infection in rats (286). Of more fundamental importance are the studies analyzing production of IFN-γ and the effects of its neutralization or ablation in model virus infections in mice and rats (219, 287–290). Generally speaking, these studies have indicated that endogenous IFN-γ is essential for adequate host defense against virus infection, i.e., for elimination of the virus following primary infection and, in some instances, also for establishment of adequate immunity against reinfection. A striking hallmark of the importance of IFN-γ as a defense mechanism against virus infections is the fact that poxviruses of diverse animal species, having coevolved with their host under pressure of endogenous IFN-γ, have acquired genetic codes to instruct infected cells to produce soluble decoy receptors for IFN-γ (291). A question that remains largely unsolved is whether the *in vivo* antiviral effects of endogenous IFN-γ are due to its direct antiviral effects on cells or to its immunomodulatory activities such as activation of NK cells, promotion of TH1- over TH2-type responses, or maturation of cytotoxic T lymphocytes. Moreover, as is the case in bacterial infections, endogenous IFN-γ can act to the detriment of the host as is evident from the observation that treatment with anti-IFN-γ antibody can

convert an aggressive into an inapparent infection with LCM virus (287). In the case of HIV, activation of monocytoid cells by IFN-γ was also found to stimulate rather than to inhibit virus replication (292, 293).

Rejection of some experimental tumors is associated with the presence of IFN-γ in the tumor tissue. That this IFN-γ contributes to this process is evident from studies with immunogenic autologous or isologous tumors, the rejection of which is abrogated by administration of neutralizing anti-IFN-γ antibodies (294, 295). Investigators have inserted the IFN-γ gene into nonimmunogenic murine tumor cells with high metastasizing potential and found that the IFN-γ secreting cells, when injected in syngeneic mice, had less ability than the parental cells to develop into tumors (296, 297); this suppression of tumorigenicity was reversed by the administration of anti-IFN-γ or anti-Lyt 2.2 antibodies. Discordant with these observations are several reports describing enhancement by IFN-γ of tumor growth or metastatic potential of experimental tumors. A metastasizing murine mammary carcinoma (TS/A-pc), productively transfected with the IFN-γ gene, was found to metastasize more extensively than the untransfected tumor line (298). Also, treatment of mice bearing Lewis lung tumors with anti-IFN-γ antibodies was found to reduce tumor outgrowth (299). *In vitro* treatment of carcinoma cells with IFN-γ prior to their inoculation in mice has been reported to enhance metastatic potential (300); the mechanism involved in this model appeared to be augmentation by IFN-γ of the tumor cells' resistance to the cytolytic effect of NK cells.

IFN-γ may be instrumental in bringing about cachexia associated with tumor development. It is generally recognized that unabated overproduction of cytokines constitutes a crucial pathogenetic element in cachexia. The cytokine that is historically most intimately associated with cachexia is TNF, also once called cachectin. IFN-γ, sometimes more so than TNF, is crucially involved in the syndrome of cachexia (301). Neutralizing antibodies against IFN-γ were shown to prevent cachexia in mice or rats carrying experimental tumors (299, 302). Moreover, acute cachexia was found to develop in nude mice inoculated with CHO cells productively transfected with the mouse IFN-γ gene but not in mice receiving the parent tumor (303).

VII. Role of IFN-γ in Immunopathology

A. IFN-γ in Delayed-Type Hypersensitivity (DTH)

IFN-γ is among the local humoral factors that were the first to be held responsible for the DTH inflammatory response. Indeed, an early finding in studies on DTH reactions was that T cells respond to antigen by production of factors, one of which was termed MAF and was later found to be identical with IFN-γ. At present, many well-defined cytokines are recognized to be involved in the DTH inflammatory response. Of particular importance as effector cytokines, are the granulocyte- and monocyte-chemotactic factors, now designated as chemokines, which are instrumental in causing cellular infiltration.

IFN-γ is likely to play a role both in the immunization process that leads to the establishment of the DTH state (afferent arm) and in the triggering events of the DTH reaction on reexposure to the antigen (efferent arm). However, experimental systems have not always allowed to clearly make this distinction, and the role of IFN-γ may be different in these different types of DTH responses. In a 4-day DTH induction model using sheep erythrocytes as an antigen in mice, exogenous IFN-γ was found to reverse inhibition of the response by anti-CD4 or anti-IL-2R antibodies (304), supporting the concept that production of IFN-γ by TH1 cells is essential for the reaction. Using a local adoptive transfer assay with Ag-specific mouse TH1 clones, administration of anti-IFN-γ antibodies was found to inhibit responses in some but not all instances (305), indicating that the positive contribution of IFN-γ to the DTH effector phase is real but can vary perhaps with specificity of the clone or with mouse strain. In the rat, administration of IFN-γ-neutralizing monoclonal antibodies was found to inhibit lymphocyte recruitment in DTH sites induced with KLH (306). Delayed-type hypersensitivity does not normally occur after immunization by injection of antigens in the anterior chamber of the eye. Transgenic mice with ectopic expression of IFN-γ in the photoreceptors of the retina, on the contrary, were found not only to have more inflammation in the eye, but also to have developed a DTH state. Thus, intraocular IFN-γ production can overcome the so-called anterior chamber-associated immune deviation (307).

Whereas these observations support the view that endogenous IFN-γ enhances DTH reactions to antigens introduced primarily into tissues, the situation may be more complex in skin contact sensitivity-type reactions. Pure IFN-γ introduced into healthy skin induces a moderate perivascular lymphohistiocytic infiltrate, an intense class II antigen expression on keratinocytes, and an apparent migration of Langerhans cells from the epidermis to the dermis (308). Similar observations were made when IFN-γ was injected in the skin of patients with lepromatous leprosy (309). In a passive transfer model in mice allowing the study of a population of T cells that suppresses DNFB contact sensitivity responsiveness, exogenous IFN-γ was found to antagonize suppressive cells, thereby identifying a pathway by which IFN-γ favors contact sensitivity (310). However, in a DNFB contact sensitivity model in rats (311), IFN-γ was found to act as a counter-regulator of the inflammatory changes: systemic administration of anti-

IFN-γ augmented ear swelling toward DNFB in sensitized animals. This effect was accompanied by reduced MHC class II antigen expression on keratinocytes. Administration of IFN-γ in this same model inhibited ear swelling if given prior to challenge but not if given later (312). The authors considered that MHC class II antigen-expressing keratinocytes are instrumental in the counterregulatory effect of IFN-γ. Such cells have indeed been shown to be able to inhibit T cell responses (160). Because exogenous IFN-γ inhibited the skin reaction when MHC class II antigen expression on keratinocytes had already taken place, it seems that it does not (or not only) act by inducing class II expression on keratinocytes, but rather (or also) by stimulating these cells to exert their purported suppressive potential.

B. IFN-γ in Organ-Specific Autoimmune Disease

IFN-γ has many ways to to intervene in both the afferent and efferent arms of autoimmune responses. It may favor the emergence of autoreactive lymphocytes by participating in lymphocyte selection in the thymic environment: the thymus contains both cells that produce IFN-γ and IFN-γ target cells. IFN-γ can also regulate the activity of autoreactive lymphocytes by affecting their clonal expansion, differentiation, regulation of cytokine production, cytotoxicity, and helper or suppressor activities (see relevant sections). Of particular potential importance is the apparent ability of IFN-γ to critically contribute to the conversion of anergic autoreactive T cells into active effectors, as illustrated by experiments with organ-specific overexpression of IFN-γ. Mice that constitutively express IFN-γ in the pancreas develop autoimmune pancreatitis (313). When such mice are additionally engineered to also express a viral antigen (*in casu* the LCMV nucleoprotein or glycoprotein antigen), they develop autoimmune diabetes more rapidly and, significantly, possess viral antigen-specific cytotoxic T lymphocytes that are absent in the single-transgenic mice (314).

In experimental animal models for autoimmune disease, administration of IFN-γ is mostly found to facilitate induction of disease or to aggravate disease manifestations. In the same or similar models, blockage of endogenous IFN-γ by administration of neutralizing antibodies reduces disease incidence or symptoms. Examples are autoimmune thyroiditis in mice (315, 316), autoimmune insulin-dependent diabetes in mice (317), and experimental autoimmune peripheral neuritis (318, 319). An autoimmune disease-promoting role for IFN-γ is by no means the general rule as will be evident from analysis of some selected model systems.

1. Autoimmunity in the CNS

Experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune uveitis in mice are reduced in incidence and severity by the administration of IFN-γ and augmented by treatment with antibodies against IFN-γ (320–324). Not only actively induced, but also passively transferred EAE is inhibited by anti-IFN-γ antibody (321), suggesting that IFN-γ exerts its disease-limiting effect by acting on the efferent rather than the afferent arm of the autoimmune process, i.e., by inhibiting the action rather than the generation of CNS antigen-reactive T cells.

During EAE in Lewis rats, IFN-γ is produced both in the CNS and peripherally (325). The effector T cells by which the disease can be transferred have been found to produce IFN-γ when confronted with MBP. In contrast, the suppressor splenic T cells found during reconvalescence of the disease fail to produce IFN-γ and inhibit IFN-γ production when added to challenged effector T cells (326). Although these data suggest that, in the early induction phase, IFN-γ favors initiation of the disease, this interpretation is difficult to reconcile with the aggravating effect of anti-IFN-γ antibodies in the mouse model.

The disease-preventing effect of IFN-γ in murine EAE invokes an immunosuppressive or anti-inflammatory pathway. One such pathway is the induction of NO (see relevant sections). However, evidence as to the effect of NO in EAE is contradictory. In adoptively transferred EAE in SJL mice the NO synthase inhibitor aminoguanidine was found to ameliorate disease parameters (327). In actively induced EAE rats, by contrast, treatment with the NO synthase inhibitor L-NMMA was found to cause aggravation of disease (S. R. Ruuls, personal communication).

2. Autoimmune Arthritis

Locally administered IFN-γ was found to promote development of collagen-induced autoimmune arthritis in mice (328), whereas systemically administered IFN-γ exerted a protective effect (329). Systemically administered IFN-γ was also found to inhibit inflammatory cell recruitment and disease signs in bacterial cell wall-induced arthritis (330). Blockage of endogenous IFN-γ by administration of anti-IFN-γ antibodies in experimental arthritis has opposing effects depending on time: early blockage tends to favor disease development, whereas late blockage exerts a protective effect (331). Clearly, IFN-γ affects the disease process in opposite directions when acting via different pathways.

IFN-γ stimulates synovial cell proliferation, an effect by which local IFN-γ in joints may aggravate disease. It should be noted, however, that in synoviocytes cultured from arthritic joints, IFN-γ reportedly antagonizes cell growth-promoting effects of TNF (332) and IL-1 (333). Local production of IL-1 and TNF in the joints is considered to be a pathogenetically important process, as these cytokines induce production by chondrocytes and synovial cells of tissue-destructive enzymes. The production of IL-1β

by synovial macrophages in rheumatoid arthritis was found to be inhibited by IFN-γ (334), suggesting that local production of IFN-γ acts as a disease-limiting factor. Furthermore, evidence indicates that IFN-γ also opposes the action of locally produced IL-1 and TNF. Thus, IFN-γ was found to inhibit metalloproteinase production and glycosaminoglycan release by cultured cartilage fragments (335). In cultured human articular chondrocytes, IFN-γ at concentrations as low as 1 unit/ml synergizes with TNF in inducing PGE production, but acts antagonistically to TNF in decreasing TNF-induced caseinase production (336): caseinase levels in culture fluid reflect those of the proteoglycanase stromelysin. In synoviocytes cultured from arthritic joints, IFN-γ was found to act antagonistically to TNF (332) and to IL-1 (333), as it inhibited cell growth, PGE2 release, and collagenase production.

Another property of IFN-γ that may be relevant to its action in autoimmune joint disease is its inhibitory effect on bone resorption in an *in vitro* system (337) and on the formation of osteoclast-like multinucleated cells in long-term human bone marrow cultures stimulated with vitamin D3 (338).

3. *Autoimmune Insulitis*

Systemic injection of streptozotocin into mice of certain strains induces insulin-dependent diabetes resembling type I diabetes occurring in man. Mononuclear cell infiltrates occur in the pancreatic islets and treatment with immunosuppressive drugs can retard disease development. Streptozotocin-induced diabetes, as assessed by hyperglycemia and body weight loss, was found to be more severe in mice that also received IFN-γ injections (339). IFN-γ treatment was also found to augment expression of MHC class I and class II antigens in the streptozotocin-challenged mice. Remarkably, IFN-γ by itself fails to induce MHC class II antigen expression in β cells, whereas it does so in most other cell types and tissues *in vivo*. The cofactor required for class II antigen expression on β cells of insulitis-affected pancreas may be TNF (116).

Another experimental model is insulin-dependent diabetes that occurs spontaneously, but with low incidence, in NOD/Wehi mice. By giving single injections of cyclophosphamide, the incidence can be increased so that in a high proportion of mice β cell destruction and hyperglycemia occur in a matter of weeks. In such mice, T cells disappear from the pancreas within 2 or 3 days after cyclophosphamide injection and then reappear in much greater numbers 1 week later. At that time, a dramatic increase occurs in expression of MHC class I protein on islet cells and on infiltrating inflammatory cells. Pretreatment of such mice with anti-IFN-γ antibodies was found to reduce the incidence and severity of the syndrome (317, 340) and also to prevent overexpression of MHC class I antigen

(341). It is considered that class I antigen overexpression is instrumental in targeting cytotoxic T cells to β cells.

Counter to expectation, administration of IFN-γ in this mouse model of diabetes did not affect blood glucose profiles. In fact, in combination with TNF-α, IFN-γ treatment was associated with a reduction in severity of islet inflammation, although this treatment caused moderate to severe pancreatitis and several other pathologic changes (342). The apparent contradiction between results obtained with IFN-γ and those with anti-IFN-γ treatment is reminiscent of a similar paradoxical observation in GVH disease models (see relevant section).

Further evidence for IFN-γ to serve a role in insulitis has come from studies with IFN-γ-transgenic mice. Expression of IFN-γ in islets of Langerhans was found to result in their inflammatory destruction (313). Another feature of these IFN-γ-transgenic mice is the presence of distended ducts indicating duct cell proliferation and islet neogenesis (343). Treatment of the mice with neutralizing anti-IFN-γ antibody was found to halt progression of the disease (344). Because no evidence of circulating IFN-γ could be found, the authors considered that the pancreatic lesions result from a local effect of IFN-γ produced in the pancreas. Lymphocytes from the transgenic mice were found to be cytotoxic for islets *in vitro* (313). Therefore, the authors proposed that islet destruction in this model is due to cytocidal effects of infiltrating lymphocytes rather than to a direct cytotoxic effect of locally produced IFN-γ. Overexpression of MHC class II antigens was noted to occur on exocrine cells. Furthermore, pancreatic endothelia of transgenic mice displayed spontaneous expression of the vascular addressins MadCam-1 (known to be required for lymphocyte homing to Peyer's patches) and MECA-79 ligand (normally located in peripheral lymph nodes); the ICAM-1/LFA-1 pair was found to be expressed on endothelial cells, pancreatic duct epithelial cells, and lymphocytes, but not on islet cells (345). These changes also occurred in IFN-γ-transgenic SCID mice indicating that they resulted directly from local IFN-γ production and not as an effect secondary to cellular infiltration. Whereas induction of vascular addressins and adhesion molecules can be considered to account for the earliest inflammatory changes in insulitis, loss of tolerance remains unexplained.

By its ability to induce nitrogen oxide production, IFN-γ, in concert with TNF, may directly contribute to islet cell death. Thus, it was shown that IFN-γ and TNF synergistically induce NO in mouse islet cells, and that accompanying cytotoxicity can be prevented by L-NMMA (346).

C. IFN-γ in Allograft Rejection and Graft Versus Host Reactions

The rejection of an allogeneic tumor by mice was found to be delayed or abrogated by pretreatment of the recipient hosts with anti-IFN-γ antibody,

suggesting that endogenous IFN-γ is crucial to allograft rejection (199). Along this line, treatment of skin allograft recipient mice with anti-IFN-γ antibody has been found to prolong rejection if the graft is MHC class II antigen-incompatible, but not if it is only MHC class I-incompatible (347). Only a small percentage of resting skin epidermal cells, i.e., Langerhans cells, express MHC class II gene products. However, after exposure to IFN-γ, all keratinocytes can become class II antigen-positive. The result was therefore interpreted to mean that, if endogenous IFN-γ contributes to the rejection of a skin allograft, it does so because it induces class II expression on keratinocytes.

The role of IFN-γ in skin allograft rejection was also studied in C3H/HEJ mice rendered hyporesponsive to B10.BR skin allografts by pretreatment with irradiated donor lymphoid cells. In this model, allograft enhancement can be achieved if the irradiated cells are injected via the portal but not via the tail vein. This state of relative anergy was found to be associated with reduced mRNA levels in lymphocytes for IFN-γ and IL-2 and increased levels of mRNA for IL-4 and IL-10. Significantly, repeated treatment with anti-IFN-γ antibody allowed graft enhancement and altered TH1/TH2 cytokine profile to be achieved with injection of irradiated lymphocytes in the tail vein (348).

In spleens of mice with experimentally induced acute GVH disease, production of IFN-γ, together with that of other cytokines, has been demonstrated (349, 350). Spleen cells of mice with GVH disease respond to mitogen stimulation with IFN-γ production when taken during the early proliferative phase of the disease and with TNF production when taken during the later destructive phase (351). In several independent studies (349, 352), the contribution of this IFN-γ to the disease manifestations has been assessed by the use of neutralizing anti-IFN-γ antibodies. These studies are unanimous in observing that blockage of IFN-γ inhibits disease development, in particular the lesions in the gut mucosa. On the other hand, as an apparent paradox, it has been reported that systemic administration of IFN-γ inhibits disease development in much the same way as anti-IFN-γ antibody does. This inhibition was associated with reduced numbers of IFN-γ-producing cells (349). Suppression of endogenous cytokine production may reflect immunosuppressive circuitry triggered by systemic and perhaps less so by locally produced IFN-γ.

Another model of GVH disease consists of inoculating neonatal mice with semiallogeneic lymphocytes, which allows for the persistence of the donor cells in the host. These cells differentiate into TH2-like cells as evident from predominance of IL-4 production over that of IL-2 and IFN-γ. As an apparent consequence, donor B cells differentiate to produce large quantities of IgE and IgG1 autoantibodies, resulting in immune deposits

and SLE-like pathology. In this model, exogenous IFN-γ was found to prevent the disease apparently by restoring the ability of the lymphocytes to produce IL-2 and IFN-γ (353).

D. IFN-γ in Nonspecific Inflammation and Generalized Cytokine Release Syndromes

Systemic administration of IFN-γ in mice can provoke a state of reduced responsiveness of tissue to aspecific inflammatory stimuli. Thus, the footpad swelling reaction to local endotoxin injection is diminished (354) and accumulation of polymorphonuclear cells in response to local injection of IL-1 into the footpad is reduced (244). These apparently anti-inflammatory effects of IFN-γ are so far unexplained and, in fact, contrast with established activities of IFN-γ in *in vitro* systems, e.g., induction of increased adhesion between endothelia and leukocytes.

In clinical immunology, many situations have become known in which exposure to infection, allergens, or other immune stimulants elicit overreactions that are often so acute and severe that they threaten life. Some of the best known examples are anaphylactic shock due to massive exposure to IgE-reactive allergens, serum sickness due to exposure to xenogeneic serum proteins, circulatory and organ failure due to gram-negative bacterial sepsis (septic shock), and toxic shock syndrome due to a staphylococcal exotoxin entering the general circulation. A recent example is the anti-CD3 antibody first-injection syndrome, an anaphylactoid state occurring in organ transplant patients who receive a large dose of anti-CD3 antibody as a means to eliminate or inactivate T cells and thus to prevent transplant rejection.

A common central element in the pathogenesis of these syndromes is the sudden and excessive release of immune mediators, such as histamine and histamine-like substances in anaphylaxis, or activated complement components in serum sickness. Normally, these mediators support the immune system in dealing with microbial or other aggressors. However, when overproduced, they cause more harm than good. The same holds true for cytokines: when inappropriately produced, they can trigger complications that have become known as "cytokine release syndromes."

In septic shock, the eliciting factor is endotoxin, which has long been recognized to trigger release of several mediators from various sources and by different pathways, including direct activation of complement and induction of degranulation of platelets. A recently recognized pathway leading to endotoxin-associated generalized reactions is the induction of cytokines. Particularly well studied is the induction by endotoxin of IL-1, TNF, IL-6, IL-12, and IFN-γ. There is experimental evidence for a role of each of these cytokines in bringing about or in counteracting the *in*

vivo changes associated with endotoxin-induced shock reactions (283). Experimental animals exposed to endotoxin produce only small quantities of IFN-γ in the circulation. Nevertheless, the case for this endogenous IFN-γ to play a critical role as a promotor of the pathological signs is very strong, as is discussed in another section.

Toxic shock syndrome toxin of *Staphylococcus* is a superantigen that associates with antigen-presenting cells and then activates a large number of T cell subsets. Such activated T cells release cytokines, including IFN-γ, that flood the circulation. A murine model system for the study of the superantigen-induced pathology consists of injecting BALB/c mice with staphylococcal enterotoxin B (SEB). These mice develop acute but transient hypoglycemia and rapid weight loss. The occurrence of these manifestations can be prevented by pretreatment with antibodies against IFN-γ (355), but equally so by antibodies against TNF-α (356), indicating that both cytokines have an important role to play. A similar situation occurs in mice injected with anti-CD3 antibody. In this model, the cytokine release syndrome manifests itself mainly by hypothermia. Both anti-TNF (357) and anti-IFN-γ antibodies (358) can prevent the symptoms.

It has been considered that IFN-γ and TNF, and perhaps additional cytokines, synergize in cytokine syndromes by forming a cascade. Support for this concept was obtained in endotoxin-injected mice: blockage of IFN-γ not only prevented disease and death but also reduced the production of TNF, suggesting that in this system endogenous IFN-γ acts as an enhancer of TNF production (284). In the SEB- or anti-CD3-injected mice, however, blockage of IFN-γ did not affect TNF release (355, 358).

Although these observations indicate that IFN-γ promotes development of cytokine release syndromes, other evidence points at its ability to also trigger a counterregulatory pathway. Thus, IFN-γ receptor-deficient strain 129 mice were found to be more sensitive to the anti-CD3-induced syndrome than the wild-type strain (197), a finding that is in apparent conflict with the results obtained with anti-IFN-γ antibodies in BALB/c mice. However, from experiments with the NO synthetase blocker, L-NMMA, it was evident that NO induction by IFN-γ provides protection against the manifestations of the syndrome. Failure of this pathway to be triggered in the IFN-γ receptor knockout mice probably accounts for their higher sensitivity.

It thus appears that in cytokine release syndromes, as in so many other situations, IFN-γ production plays a Janus role, promoting manifestations via one set of pathways and counteracting them via others. Depending on the test system, or clinical situation, IFN-γ may display one or the other of its Janus faces.

VIII. Final Thoughts

What are the most salient issues of recent and current IFN-γ research?

Especially obvious is the progress in our understanding of the cellular and molecular biological aspects of IFN-γ action. The once-elusive IFN-γ receptor has yielded most of its secrets. Investigation of IFN-γ signal transduction has brought to light a new pathway, i.e., the STAT molecules, which is used by many different cytokines.

New insight has been generated in the biology of IFN-γ production. The NK cell population has been identified as a source of IFN-γ that appears to be as important as the T cells. One of the latter-day cytokines, IL-12 or NK cell-stimulating factor, owes much of its importance to its IFN-γ-inducing capacity. Integration of IFN-γ in the cytokine network has gained more substance by clarification of interactions with other cytokines, especially those that antagonize IFN-γ production and/or action: IL-10, IL-4, and TGF-β.

Generation of nitrogen oxide has been pinpointed as a crucial mediator of cellular and organ responses to IFN-γ, e.g., cytocidal effects, enhanced intracellular microbial killing and inflammation, but also immunosuppressive counterregulation. The fact that nitrogen oxide is involved in a wide spectrum of physiological and pathological processes and that most cells of the body have the potential to respond to IFN-γ makes it seem likely that IFN-γ will be found to be involved in an even wider range of tissue reactions than is presently recognized. Nevertheless, evidence that IFN-γ plays a role in normal physiology is still lacking. Especially promising from this point of view is the availability of laboratory mouse strains that are deficient in either the production of IFN-γ or in the expression of IFN-γ receptor molecules. To date, however, experiments employing these animals have little more than confirmed what was already known from other approaches, i.e., the important role of IFN-γ as a irreplaceable factor in host defense against infection and as a wrongdoer in inflammation and autoimmune disease.

Identification of IFN-γ as a mediator of tissue reactions in inflammatory and autoimmune disease has generated a cogent quest for usable antagonists, e.g., humanized monoclonal antibodies, soluble receptors, or antagonistic cytokines. It seems likely that such IFN-γ antagonists, if made available, will find their way into the clinic for diseases with an autoimmune component such as lupus erythematosus, multiple sclerosis, Crohn's disease, and others. Intriguing, however, is the unexpected observation that IFN-γ can, in some animal models, inhibit rather than promote autoimmune-type diseases, e.g., experimental autoimmune encephalomyelitis and graft versus host disease.

IX. Notes Added in Proof

Over the few months since submission of this chapter, numerous publications in relation to IFN-γ have appeared. Here, a selection representative of the current trend in IFN-γ research is given and commented upon.

Our conception of the early events following interactions of IFN-γ with its receptor have been further refined. With many cytokines, e.g., IL-2, the number of receptors present on cells is downregulated by exposure to the cytokine. In the case of IFN-γ an aberrant type of receptor downregulation occurs in some cells (359), in particular murine $CD4^+$ T cells, but not in others, e.g., mouse L929 fibroblast-like cells. Downregulation affects only the β-chain which plays no part in ligand binding but does transmit the signal to the cytoplasm. This mechanism is held responsible for the virtual absence of β-chains in cultured murine TH1 cells which produce IFN-γ themselves (194,359). However, β-chain downregulation is not inherent to TH1 cells since both TH1 and TH2 cells do retain the β-chain in IFN-γR knock-out mice.

Research on membrane signaling has refined the model for interaction of the receptor with intracellular proteins: JAK1 and JAK2 are associated, respectively, with the α- and β-chains of the receptor. Clustering of the receptor chains results in phosphorylation of both JAKs, in activation of JAK2 activity and in association with STAT1α (360). Furthermore, a transcription activation factor, γRF-1, which associates with the γRE-1 sequence in the *mig* gene, has been isolated and characterized (361).

The molecular mechanism of induction of class II MIIC gene products by IFN-γ MHC has been clarified to some extent. The promoter regions of class II MHC and invariant chain genes contain several regulatory elements. However, these elements are only indirectly controlled by IFN-γ, through an IFN-γ-induced transactivator protein called CIITA (class II transactivator). The CIITA gene has been isolated in the course of studies on the defects in patients suffering from bare lymphocyte syndromes (362). CIITA is required for constitutive expression of class II MHC genes in professional antigen-presenting cells, but is also involved in IFN-γ-induced expression in cells which are constitutively class II-negative. IFN-γ induces expression of CIITA mRNA before induction of class II MHC mRNA molecules. Moreover, CIITA mRNA induction is not blocked by cycloheximide, whereas induction of MHC Class II mRNA itself does require protein synthesis. Induction of CIITA mRNA does require JAK1 activity but is not dependent on expression of IRF-1. There is no evidence that CIITA is a DNA-binding factor (363,364).

The important role of NK cells as producers of IFN-γ has received further support. IL-15 is a recently characterized monocyte-derived cytokine which, like IL-12, activates NK cells and stimulates them to produce IFN-γ. In cocultures of monocytes and NK cells, the induction of IFN-γ by LPS was found to be abrogated by inclusion of neutralizing anti-IL-15 antibody, indicating that endogenous production of IL-15 is essential for IFN-γ production in this system (365). In a study on the mechanism of natural resistance to *Trypanosoma cruzi infection*, it was found that depletion of NK cells from mouse splenocyte cultures drastically reduces production of IFN-γ subsequent to parasite infection. In addition, *in vivo* depletion of NK cells by the use of anti-NK cell monoclonal antibody reduced natural resistance against the parasite (366). The NK cell-stimulating cytokine IL-12 was found to protect normal but not IFN-γ-gene knock-out mice against infection with *Mycobacterium tuberculosis* (367). Similarly, the protective effect of IL-12 against *Histoplasma capsulatum* infection in mice was found to be abrogated by treatment of the animals with anti-IFN-γ antibody (368).

Treatment with anti-IFN-γ antibody was shown to protect normal mice against a lethal dose of live staphylococci; this effect was associated with increased bacterial counts in organs early after infection, but reduced counts at later times (369). Inoculation of IFN-γR knock-out mice with a TSST-1-producing staphylococcus strain produced more frequent and more severe arthritis than was found in the wild-type counterparts. In addition, severe sepsis with high mortality occurred early after infection; at later time points the wild-type counterparts had higher mortality than did the knock-outs (370).

The contribution of IFN-γ to overall immunosuppression accompanying certain infections has been further documented. Inhibition of splenocyte-proliferative responses in mice acutely infected with *Toxoplasma* was found to be relieved by addition of anti-IFN-γ antibody to the culture medium (371). Concomitantly, anti-IFN-γ antibody-treated cultures produced less nitrite, implicating IFN-γ-induced NO as a factor in the immunosuppression. A similar situation was found to occur in mice infected with *Mycobacterium avium* (372).

The detrimental role of IFN-γ in EAE has been further documented by the observation that the actively induced disease is more severe in IFN-γ knock-out mice (373). In collagen-induced arthritis in mice, early or mid-term treatment with anti-IFN-γ was found to inhibit disease development, while late treatment was either without effect or aggravated the symptoms (374).

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Role of the CD28–B7 Costimulatory Pathways in T Cell-Dependent B Cell Responses

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I. Introduction

The cellular interactions neccessary for T cell-dependent (Td) B cell activation and generation of a humoral immune response involve the interaction of a complex array of cell surface molecules and soluble mediators. As a result of these interactions, biochemical signals are delivered to both the antigen-specific Th cell and to the antigen-specific B cell leading to the subsequent proliferation and differentiation of both cells. The Td B cell immune response requires antigen recognition by both Th cells and B cells, but T cell receptor (TcR) and B cell receptor (BcR) occupancy, although necessary, is insufficient to generate all of the signals required for the induction of a primary antibody response. Additional signals, costimulatory signals, are also required. Costimulatory signals are neither antigen-specific nor MHC restricted and arise from the coligation of counterreceptors on the cell surface of T cells and B cells or professional antigen-presenting cells (APCs). Costimulatory signals are discrete from but complementary to TcR- or BcR-derived signals. In certain situations, T cells or B cells that encounter their antigens in the absence of costimulation may be anergized or deleted.

II. Two-Signal Model of T Cell Activation

The two-signal model of lymphocyte activation was initially proposed by Bretscher and Cohn (1970). This model proposed that activation of lymphocytes requires delivery of two distinct signals, neither one of which is sufficient to activate fully. In its current form, the two-signal model specifies that antigen-specific induction of proliferation and full differentiation of T or B lymphocytes requires a first signal delivered through the TcR or BcR by antigen and a second, or costimulatory signal, delivered through receptors that are distinct from the antigen-specific TcR and BcR. This model predicts that at least three different outcomes can result from the interaction of a T cell with an APC: TcR engagement by specific antigen in the presence of costimulatory ligands on the APC leads to

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